IB/03/3163

Rec'd PCT/PTO 06 DEC 2004

PRV

PATENT- OCH REGISTRERINGSVERKET
Patentavdelningen

IB03/3163

Intyg
Certificate

Härmed intygas att bifogade kopior överensstämmer med de handlingar som ursprungligen ingivits till Patent- och registreringsverket i nedannämnda ansökan.

This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.

REC'D 10 SEP 2003
WIPO PCT

(71) *Sökande* *CePeP AB, Stockholm SE*
Applicant (s)

(21) *Patentansökningsnummer* *0201863-8*
Patent application number

(86) *Ingivningsdatum* *2002-06-18*
Date of filing

Stockholm, 2003-09-01

För Patent- och registreringsverket
For the Patent- and Registration Office

Hjördis Segerlund

Hjördis Segerlund

Avgift
Fee *170:-*

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

BEST AVAILABLE COPY

PATENT- OCH REGISTRERINGSVERKET
SWEDEN

Postadress/Adress
Box 5055
S-102 42 STOCKHOLM

Telefon/Phone
+46 8 782 25 00
Vx 08-782 25 00

Telex
17978
PATOREG S

Telefax
+46 8 666 02 86
08-666 02 86

Cell-Penetrating Peptides

FIELD OF THE INVENTION

The present invention relates to a method for predicting or designing, detecting, and/or verifying a novel cell-penetrating peptide (CPP) and to a method for using said new CPP and/or a novel usage of a known CPP for an improved cellular uptake of a cellular effector, coupled to said CPP. Furthermore, the present invention also relates to a method for predicting or designing, detecting and/or verifying a novel cell-penetrating peptide (CPP) that mimics cellular effector activity and/or inhibits cellular effector activity. The present invention additionally relates to the use of said CPP for treating and/or preventing a medical condition and to the use of said CPP for the manufacture of a pharmaceutical composition for treating a medical condition.

BACKGROUND OF THE INVENTION

A number of techniques have been developed to deliver different cellular effectors into cells. The majority of these techniques are invasive, like electroporation or microinjection. Liposome encapsulation and receptor mediated endocytosis are milder methods, but they unfortunately suffer from serious drawbacks, in particular, low delivery yield.

The established view in cellular biology dictates that the cellular internalisation of hydrophilic macromolecules can only be achieved through the classical endocytosis pathway. However, in the past five years, several peptides have been demonstrated to translocate across the plasma membrane of eukaryotic cells by a seemingly energy-independent pathway. These peptides are defined as cell-penetrating peptides (CPPs) and have been used successfully for intracellular delivery of macromolecules with molecular weights several times greater than their own. (M. Lindgren et al, 2000, Cell-penetrating peptides, TIPS, Vol. 21, pg. 99-103)

Cellular delivery using these cell-penetrating peptides offers several advantages over conventional techniques. It is non-invasive, energy-independent, is efficient for a broad range of cell types and can be applied to cells en masse. Furthermore, it has been found that for certain types of CPPs, cellular internalisation occurs at 37°C as well as 4°C and that it can not be saturated. Also, the internalisation seems not to require a chiral receptor protein, since no enantiomeric discrimination is observed.

Until recently, transport of hydrophilic macromolecules into the cytoplasmic and nuclear compartments of living cells without disrupting the plasma membrane seemed a far-off goal. Because of their low biomembrane permeability and their relatively rapid degradation, polypeptides and oligonucleotides were generally considered to be of limited therapeutic value. This is an obstacle in both biomedical research and the pharmaceutical industry.

An even more difficult, although very important task is to deliver hydrophilic macromolecules across the blood–brain barrier. Several methods have been envisaged to overcome this hurdle. Nevertheless, they all suffer from limitations, such as their effectiveness being restricted to a subset of molecules, or to low yield. However, recent reports suggest that CPPs might be able to transport macromolecules across the blood–brain barrier.

Another important area for desired delivery of effectors is nuclear import, wherein, in general, it has been found that the signal sequence must contain some positively charged (basic) residues (M. Lindgren et al., Trends Pharmacol. Sci. 21 (2000) 99-103). It seems that such charged amino acids might also be required for plasma membrane translocation.

Today, a diversity of cell-penetrating peptides, CPPs, is known. Several peptides have been demonstrated to translocate across the plasma membrane of eukaryotic cells by a seemingly energy-independent pathway. Thus, cell-penetrating peptides might be used as delivery vectors for pharmacologically interesting substances, such as peptides, proteins, oligonucleotides, antisense molecules, as well as research tools.

Of particular interest among CPPs are those peptides which have low lytic activity. These translocating peptides, also known as Trojan peptides (D. Derossi et al., Trends Cell Biol. 8 (1998) 84-87), have been applied as vectors for the delivery of hydrophilic biomolecules and drugs into cytoplasmic and nuclear compartments of cells, both *in vivo* and *in vitro* (M. Lindgren et al., Trends Pharmacol. Sci. 21 (2000) 99-103). When covalently linked with a cargo, including polypeptides and oligonucleotides with many times their own molecular mass, these peptides are still able to translocate.

Examples of useful transport peptides are sequences derived from homeodomains of certain transcription factors, as well as so-called Tat-derived peptides and peptides based on signal sequences. The first of the homeodomain-derived translocating peptides was penetratin, denoted pAntp, with a sequence corresponding to the 16 residues of the third α-helix (residues 43-58) from the Antennapedia homeodomain protein of Drosophila (D. Derossi et al., J. Biol. Chem. 269 (1994) 10444-10450; A. Prochiantz, Ann. NY Acad. Sci. 886 (1999) 172-179). The pAntp peptide retains its membrane translocation properties and has therefore been proposed to be a universal intercellular delivery vector (D. Derossi et al., Trends Cell Biol. 8 (1998) 84-87).

Purely synthetic or chimeric peptides have also been designed, as reviewed in (D. Derossi et al., Trends Cell Biol. 8 (1998) 84-87, M. Lindgren et al., Trends Pharmacol. Sci. 21 (2000) 99-103).

Transportan, e.g., a non-natural peptide, is able to deliver an antibody molecule with molecular mass of about 150 kDa over the plasma membrane, although Transportan itself is only a 3 kDa peptide. Transportan and penetratin were demonstrated to deliver a non-

natural DNA analogue, PNA (peptide nucleic acid) into cytoplasm and nuclei of cells in culture.

Although their astonishing transport capability has put CPPs into the focus of scientific interest for the last 5 years, the complete mechanism of translocation for the different CPPs is still unknown. For instance, it is not known whether any particular secondary structure has to be induced in order to allow (energetically) a translocation, involving a concomitant transient membrane destabilization. It is clear, however, that the molecular details of the peptide-membrane interactions must be of fundamental importance for the translocation process.

The mechanism and requirements for internalisation have been studied on interactions between amphipathic α-helical peptides and lipid (bi)layers. The results of these studies often suggest tryptophan to be responsible for internalisation of a peptide, but although aromatic amino acids may be preferred in CPP sequences, they are not absolutely necessary for cell penetration.

Apart from the cell penetration capability, little correlation of structure or behaviour has been found between CPPs. Up to now, CPPs have thus not been designed in a rational manner, but have been found serendipitously. However, the sequences of CPPs published so far have a positive net-charge as the only common feature, giving a starting point for the prediction of CPP functionality in a given peptide sequence. Clearly, though, all sequences with a positive net-charge cannot be cell-penetrating, indicating that further restrictions are needed to select CPPs with any certainty.

The present invention for the first time provides a novel general principle for predicting, designing, detecting and/or verifying a cell-penetrating peptide and/or a non-peptide analogue thereof, characterised by application of a novel prediction/selection criteria, optionally in combination with a method for testing the cellular penetration capacity of said found CPP.

SUMMARY

The present invention relates to a method for predicting, detecting, designing and/or verifying a cell-penetrating peptide (CPP) and/or a non-peptide analogue thereof, characterised by application of a novel prediction/selection criteria, optionally in combination with a method for testing the cellular penetration capacity of said found CPP *in vitro* and *in vivo*.

A unifying aspect of the invention is thus directed to a method of identifying a cell penetrating amino acid fragment, comprising assessing the bulk property value Z_Σ of said sequence, Z_Σ comprisisng at least 5 individual average interval values $Z_{\Sigma 1}$; $Z_{\Sigma 2}$; $Z_{\Sigma 3}$; $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$, wherein $Z_{\Sigma 1}$, $Z_{\Sigma 2}$, $Z_{\Sigma 3}$, $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$ are average values of the respective descriptor values for the residues in said amino acid sequence, calculated with the formula

$$Z_{\Sigma x} = (Z_{xres1} + Z_{xres2} \ldots + Z_{xresn})/n$$

Z_{xresy} being the respective descriptor value for amino acid residue y comprised in the selected candidate fragment, and wherein the descriptor value of each residue corresponds to a Z_1, Z_2, Z_3, Z_4, and Z_5 descriptor value in a descriptor value scale, and wherein a cell penetrating fragment is characterised by having a Z_Σ bulk property value essentially consisting of individual average interval values, wherein $Z_{\Sigma 1}<0.2$; $Z_{\Sigma 2}<1.1$; $Z_{\Sigma 3}<-0.49$; $Z_{\Sigma 4}<0.33$; and $Z_{\Sigma 5}<0.95$ and $Z_{\Sigma 5}>0.12$.

The invention further relates to a method for using said novel CPP and/or to a novel and improved usage of a known CPP for improved cellular uptake of a cellular effector coupled to said CPP, and to a method for predicting, detecting, designing and/or verifying a novel cell-penetrating peptide (CPP) that has cellular effector activity itself. Furthermore, the present invention also relates to the use of said CPP and/or said improved usage of a known CPP for treating and/or preventing a medical condition, and/or for the manufacture of a pharmaceutical composition for treating a medical condition.

DETAILED DISCLOSURE

The present invention for the first time discloses a novel general principle for predicting, designing, detecting and/or verifying a cell-penetrating peptide and/or a non-peptide analogue thereof, characterised by application of a novel prediction/selection criterium, optionally in combination with a method for *in vivo* or *in vitro* testing the cellular penetration capacity of said found CPP, either derived from a random sequence or a naturally occurring protein.

Evaluation of predictors relevant for the function of cell-penetrating peptides

In most peptide quantitative structure activity relationship studies (QSAR), a set of dimensionless values is used to describe a composite of the physical characteristics of the amino acids. In the classical literature, 3 values, Z_1, Z_2 and Z_3 are used for this purpose. Recently Wold and colleagues expanded this descriptor set with 2 more: Z_4 and Z_5; and produced descriptor scales covering 87 natural and non-natural amino acids (Sandberg, M., Eriksson, L., Jonsson, J., Sjöström, M., and Wold, S., New chemical descriptors relevant for the design of biologically active peptides. A multivariate characterization of 87 amino acids, J. Med. Chem., 41, 2481 (1998).

The novel methods described in the present application comprise using said expanded QSAR descriptor scales for the evaluation of CPP functionality. Said new methods thus open a fast and reliable way to the production of CPPs, consisting of natural as well as non-natural building blocks. Moreover, a rigorous quantification of CPP uptake in a physical test as disclosed herein, even enables a QSAR model for tissue specificity.

Table 1A.
Descriptor Scales for the Characterized Coded and Non-Coded Amino Acids

no.	abbrev	name[a]	Z_1	Z_2	Z_3	Z_4	Z_5
1	Ala	alanine	0.24	-2.32	0.60	-0.14	1.30
2	Arg	arginine	3.52	2.50	-3.50	1.99	-0.17
3	Asn	asparagine	3.05	1.62	1.04	-1.15	1.61
4	Asp	aspartic acid	3.98	0.93	1.93	-2.46	0.75
5	Cys	cysteine	0.84	-1.67	3.71	0.18	-2.65
6	Gln	glutamine	1.75	0.50	-1.44	-1.34	0.66

7	Glu	glutamic acid	3.11	0.26	-0.11	-3.04	-0.25
8	Gly	glycine	2.05	-4.06	0.36	-0.82	-0.38
9	His	histidine	2.47	1.95	0.26	3.90	0.09
10	Ile	isoleucine	-3.89	-1.73	-1.71	-0.84	0.26
11	Leu	leucine	-4.28	-1.30	-1.49	-0.72	0.84
12	Lys	lysine	2.29	0.89	-2.49	1.49	0.31
13	Met	methionine	-2.85	-0.22	0.47	1.94	-0.98
14	Phe	phenylalanine	-4.22	1.94	1.06	0.54	-0.62
15	Pro	proline	-1.66	0.27	1.84	0.70	2.00
16	Ser	serine	2.39	-1.07	1.15	-1.39	0.67
17	Thr	threonine	0.75	-2.18	-1.12	-1.46	-0.40
18	Trp	tryptophan	-4.36	3.94	0.59	3.44	-1.59
19	Tyr	tyrosine	-2.54	2.44	0.43	0.04	-1.47
20	Val	valine	-2.59	-2.64	-1.54	-0.85	-0.02
21	Acpa	aminocaprylic acid	-4.38	1.92	2.14	-2.61	-4.93
22	Aecys	*(S)-2-aminoethyl-L-cysteine·HCl*	3.03	2.60	0.50	2.65	-1.55
23	Afa	aminophenylacetate	-3.51	2.93	2.94	1.17	1.22

24	Aiba	-aminoisobytyric acid	-1.33	-2.80	-0.61	-0.55	0.40
25	Aile	*alloisoleucine*	-4.09	-1.28	-1.40	-0.63	0.94
26	Alg	L-*allylglycine*	-2.31	-1.35	-0.05	0.05	1.25
27	Aba	aminobutyric acid	-1.22	-2.44	-0.38	-0.51	0.65
28	Aphe	*p-aminophenylalanine*	-0.62	3.28	-0.11	3.24	-1.51
29	Bal	*-alanine*	2.16	-6.54	-4.46	-2.66	-5.93
30	Brphe	*p*-bromophenylalanine	-5.62	3.18	0.29	0.54	-1.10
31	Cha	cyclohexylalanine	-6.26	0.30	-2.58	-0.67	1.01
32	Cit	citrulline	1.31	1.47	-2.76	-2.10	0.42
33	Clala	-chloroalanine	-0.66	0.30	2.65	-0.47	1.92
34	Cle	cycloleucine	-2.95	-2.16	-1.66	-0.65	0.19
35	Clphe	*p*-chlorophenylalanine	-5.31	2.66	0.99	0.02	-1.76
36	Cya	cysteic acid	4.20	3.59	3.76	-5.09	-1.36

37	Dab	2,4-diaminobutyric acid	3.69	-0.53	-0.24	1.03	-0.15
38	Dap	2,3-diaminopropionic acid	4.34	-0.54	0.96	1.04	0.24
39	Dhp	3,4-dehydroproline	-1.24	0.40	2.50	1.48	1.53
40	Dhphe	3,4-dihydroxyphenylalanine	-0.45	3.32	-0.07	-0.33	-1.95
41	Fphe	*p*-fluorophenylalanine	-4.58	2.26	1.28	-0.70	-1.58
42	Gaa	D-*glucoseaminic acid*	4.90	3.91	-1.98	-4.18	0.89
43	Hag	homoarginine	2.70	3.06	-4.15	2.32	-0.46
44	Hlys	*hydroxylysine·HCl*	3.98	1.67	-2.51	0.32	0.08
45	Hnvl	DL-*hydroxynorvaline*	-0.85	-1.08	-1.10	-1.73	-0.04
46	Hog	*homoglutamine*	1.33	1.19	-2.14	-1.61	0.59
47	Hoph	*homophenylalanine*	-5.86	2.95	0.37	1.03	0.32
48	Hos	homoserine	0.93	-0.71	-0.01	-1.58	0.94

49	Hpr	hydroxyproline	-0.24	2.27	2.47	0.18	2.94
50	Iphe	*p-iodophenylalanine*	-6.23	6.88	3.01	1.52	1.05
51	Ise	*isoserine*	3.78	2.82	2.55	0.27	2.96
52	Mle	*-methylleucine*	-5.40	-2.07	-2.86	-1.15	-0.27
53	Msmet	DL-*methionine-s-methylsulfoniumchloride*	1.22	1.89	-0.91	3.75	-1.25
54	1Nala	3-(1-naphthyl)alanine	-5.67	6.31	3.43	3.51	-0.47
55	2Nala	3-(2-naphthyl)alanine	-6.48	6.37	2.81	3.02	-0.49
56	Nle	norleucine (or 2-aminohexanoic acid)	-4.33	-1.30	-1.54	-0.85	0.74
57	Nmala	*N*-methylalanine	-1.30	-3.13	-0.65	0.04	-0.16
58	Nva	norvaline (or 2-aminopentanoic acid)	-3.08	-1.76	-0.98	-0.68	0.87
59	Obser	*O*-benzylserine	-5.20	2.54	-0.60	0.32	-0.48

60	Obtyr	*O*-benzyltyrosine	-7.71	7.33	-1.81	2.39	0.11
61	Oetyr	*O*-ethyltyrosine	-5.62	3.33	-0.75	0.71	-1.17
62	Omser	*O*-methylserine	-1.02	-0.30	0.36	-0.97	1.70
63	Omthr	*O*-methylthreonine	-1.75	-1.63	-1.55	-1.60	-0.20
64	Omtyr	*O*-methyltyrosine	-4.28	3.05	-0.03	0.72	-1.11
65	Orn	ornithine	3.09	0.17	-1.85	1.46	0.42
66	Pen	penicillamine	0.15	-0.76	0.42	0.67	-2.79
67	Pga	pyroglutamic acid	-3.56	2.88	2.82	1.09	3.10
68	Pip	pipecolic acid	-2.66	-2.29	-1.57	0.20	-0.39
69	Sar	sarcosine	0.30	-3.55	-0.09	0.29	-0.35
70	Tfa	*3,3,3-trifluoroalanine*	-1.47	1.11	3.66	-4.70	2.13
71	Thphe	6-*hydroxydopa*	1.29	5.13	0.89	-0.93	-2.06
72	Vig	L-*vinylglycine*	-0.81	1.17	3.54	1.20	3.43

73	Aaspa	(-)-(2*R*)-2-amino-3-(2-aminoethylsulfonyl)propanoic acid dihydrochloride	5.35	6.24	2.92	-1.44	-2.26
74	Ahdna	(2*S*)-2-amino-9-hydroxy-4,7-dioxanonanoic acid	-1.40	3.33	-2.51	-2.81	1.96
75	Ahoha	(2*S*)-2-amino-6-hydroxy-4-oxahexanoic acid	0.05	1.17	-0.74	-1.96	1.64
76	Ahsopa	(-)-(2*R*)-2-amino-3-(2-hydroxyethylsulfonyl)propanoic acid	3.01	5.82	3.85	-3.86	-1.72

Using the expanded descriptor scales as listed in table 1A, the inventors assembled the 5 individual average interval values $Z_{\Sigma 1}$; $Z_{\Sigma 2}$; $Z_{\Sigma 3}$; $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$ of 4 known cell-penetrating peptides (CPPs): transportan, penetratin, pVEC and MAP; and averaged over the total number of amino acids in the sequence.

$Z_{\Sigma 1}$, $Z_{\Sigma 2}$, $Z_{\Sigma 3}$, $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$ average values of the respective descriptor values for the residues in said amino acid sequence, calculated with the formula

$$Z_{\Sigma x} = (Z_{xres1} + Z_{xres2} \ldots + Z_{xresn})/n$$

obtained from the training set are listed in Table 1B.

Table 1B: Descriptor values for the QSAR training set

Name	z_1	z_2	z_3	z_4	z_5
Transportan	-0.728	-0.992	-0.575	-0.308	0.64
pVEC	0.191	-0.118	-0.499	-0.950	0.881
penetratin	0.157	1.073	-0.586	0.167	0.296
MAP	-0.948	-1.03	-1.071111	0.087	1.08

Consequently, the inventors were able to determine that a cell penetrating amino acid fragment based on its Z_Σ bulk property value is characterised by having a Z_Σ bulk property value essentially consisting of individual average interval values, wherein $Z_{\Sigma 1}<0.2$; $Z_{\Sigma 2}<1.1$; $Z_{\Sigma 3}<-0.49$; $Z_{\Sigma 4}<0.33$; and $Z_{\Sigma 5}<0.95$ and $Z_{\Sigma 5}>0.12$.

The present invention thus discloses a first method for predicting, detecting and/or verifying a potential cell-penetrating peptide, comprising obtaining the amino acid sequence of a protein or peptide, selecting an amino acid sequence of at least one candidate fragment and assessing the bulk property value Z_Σ of said sequence, Z_Σ comprisisng at least 5 individual average interval values $Z_{\Sigma 1}$; $Z_{\Sigma 2}$; $Z_{\Sigma 3}$; $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$, wherein $Z_{\Sigma 1}$, $Z_{\Sigma 2}$, $Z_{\Sigma 3}$, $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$ are average values of the respective descriptor values for the residues in said amino acid sequence, calculated with the formula

$$Z_{\Sigma x}= (Z_{xres1}+Z_{xres2}...+Z_{xresn})/n$$

Z_{xresy} being the respective descriptor value for amino acid residue y comprised in the selected candidate fragment, and wherein the descriptor value of each residue corresponds to a Z_1, Z_2, Z_3, Z_4, and Z_5 descriptor value in a descriptor value scale as listed in **table 1A**, and identifying a cell penetrating fragment from said at least one candidate fragment(s) based on its Z_Σ bulk property value. A cell penetrating fragment is herein characterised by having a Z_Σ bulk property value essentially consisting of individual average interval values, wherein $Z_{\Sigma 1}<0.2$; $Z_{\Sigma 2}<1.1$; $Z_{\Sigma 3}<-0.49$; $Z_{\Sigma 4}<0.33$; and $Z_{\Sigma 5}<0.95$ and $Z_{\Sigma 5}>0.12$. Optionally said cell-penetrating capacity of said identified peptide or protein and/or said fragment is further verified by *in vitro* and/or *in vivo* methods.

As has been demonstrated for several of the serendipitously found CPPs, as for e.g. transportan, penetratin and tat, truncation of the original sequence can still give an active CPP. This indicates that the previously found CPPs might contain a shorter sequence acting as the transporter "motor". Taking this into account, a second aspect of the invention is directed to a method for checking cellular penetration properties of a peptide, comprising the steps of obtaining the amino acid sequence of the peptide, assessing the bulk property value Z_Σ of said sequence, Z_Σ comprisisng at least 5 individual average interval values $Z_{\Sigma 1}$; $Z_{\Sigma 2}$; $Z_{\Sigma 3}$; $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$, wherein $Z_{\Sigma 1}$, $Z_{\Sigma 2}$, $Z_{\Sigma 3}$, $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$ are average values of the respective descriptor values for the residues in said amino acid sequence, calculated with the formula

$$Z_{\Sigma x} = (Z_{xres1} + Z_{xres2} \ldots + Z_{xresn})/n$$

Z_{xresy} being the respective descriptor value for amino acid residue y comprised in the selected candidate fragment, and wherein the descriptor value of each residue corresponds to a Z_1, Z_2, Z_3, Z_4, and Z_5 descriptor value in a descriptor value scale as listed in **table 1A**, and checking the cell penetrating properties of said peptide based on its Z_Σ bulk property value, wherein a cell penetrating fragment is characterised by having a Z_Σ bulk property value essentially consisting of individual average interval values, wherein $Z_{\Sigma 1}<0.2$; $Z_{\Sigma 2}<1.1$; $Z_{\Sigma 3}<-0.49$; $Z_{\Sigma 4}<0.33$; and $Z_{\Sigma 5}<0.95$ and $Z_{\Sigma 5}>0.12$, synthesizing or isolating a peptide comprising the amino acid sequence of said identified cell-penetrating peptide, and optionally verifying the protein-mimicking functionality and/or the cell-penetrating capacity of the synthesized or isolated peptide by *in vitro* and/or *in vivo* methods.

Also comprised in the present invention is a method for producing a cell penetrating and functional protein-mimicking peptide, essentially comprising the steps of selecting a functional protein of interest, obtaining the amino acid sequence of said selected protein, selecting the amino acid sequence of at least one candidate fragment corresponding to an intracellular part of said protein, assessing the bulk property value Z_Σ of said sequence, Z_Σ comprisisng at least 5 individual average interval values $Z_{\Sigma 1}$; $Z_{\Sigma 2}$; $Z_{\Sigma 3}$; $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$, wherein $Z_{\Sigma 1}$, $Z_{\Sigma 2}$, $Z_{\Sigma 3}$, $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$ are average values of the respective descriptor values for the residues in said amino acid sequence, calculated with the formula

$$Z_{\Sigma x} = (Z_{xres1} + Z_{xres2} \ldots + Z_{xresn})/n$$

Z_{xresy} being the respective descriptor value for amino acid residue y comprised in the selected candidate fragment, and wherein the descriptor value of each residue corresponds to a Z_1, Z_2, Z_3, Z_4, and Z_5 descriptor value in a descriptor value scale as listed in **table 1A**, and identifying a cell penetrating fragment from said at least one candidate fragment(s) based on its Z_Σ bulk property value, wherein a cell penetrating fragment is characterised by having a Z_Σ bulk property value essentially consisting of individual average interval values, wherein $Z_{\Sigma 1}<0.2$; $Z_{\Sigma 2}<1.1$; $Z_{\Sigma 3}<-0.49$; $Z_{\Sigma 4}<0.33$; and $Z_{\Sigma 5}<0.95$ and $Z_{\Sigma 5}>0.12$. Finally, synthesizing or isolating a peptide comprising the amino acid sequence of said identified cell-penetrating peptide, and optionally, verifying the protein-mimicking functionality and/or the cell-penetrating capacity of the synthesized or isolated peptide by *in vitro* and/or *in vivo* methods.

Another, equally preferred, embodiment of the present invention relates to a method for producing an artificial cell penetrating and/or an artificial cell penetrating and functional protein-mimicking peptide, comprising the steps of designing at least one artificial peptide and/or peptide fragment, assessing the bulk property value Z_Σ of the amino acid sequence of said artificial peptide or peptide fragment, Z_Σ comprisisng at least 5 individual average interval values $Z_{\Sigma 1}$; $Z_{\Sigma 2}$; $Z_{\Sigma 3}$; $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$, wherein $Z_{\Sigma 1}$, $Z_{\Sigma 2}$, $Z_{\Sigma 3}$, $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$ are average

values of the respective descriptor values for the residues in said amino acid sequence, calculated with the formula

$$Z_{\Sigma x} = (Z_{xres1} + Z_{xres2} \ldots + Z_{xresn})/n$$

Z_{xresy} being the respective descriptor value for amino acid residue y comprised in the selected candidate fragment, and wherein the descriptor value of each residue corresponds to a Z_1, Z_2, Z_3, Z_4, and Z_5 descriptor value in a descriptor value scale as listed in **table 1A**, and checking the cell penetrating properties of said artificial peptide and/or peptide fragment based on its Z_Σ bulk property value, wherein a cell penetrating fragment is characterised by having a Z_Σ bulk property value essentially consisting of individual average interval values, wherein $Z_{\Sigma 1}<0.2$; $Z_{\Sigma 2}<1.1$; $Z_{\Sigma 3}<-0.49$; $Z_{\Sigma 4}<0.33$; and $Z_{\Sigma 5}<0.95$ and $Z_{\Sigma 5}>0.12$. Further synthesizing said peptide and/or peptide fragment comprising the amino acid sequence identified as cell penetrating, and optionally verifying the protein-mimicking functionality and/or the cell-penetrating capacity of the synthesized peptide and/or peptide fragment by *in vitro* and/or *in vivo* methods.

In the present context, a cell penetrating fragment is characterised by having a Z_Σ bulk property value essentially consisting of individual average interval values, wherein most preferably $Z_{\Sigma 1}<0.2$; $Z_{\Sigma 2}<1.1$; $Z_{\Sigma 3}<-0.49$; $Z_{\Sigma 4}<0.33$; and $Z_{\Sigma 5}<0.95$ and $Z_{\Sigma 5}>0.12$. In alternative embodiments of the invention, said individual average values can comprise
$Z_{\Sigma 1}<0.3$, such as $Z_{\Sigma 1}<0.21$, $Z_{\Sigma 1}<0.22$, $Z_{\Sigma 1}<0.23$, $Z_{\Sigma 1}<0.24$, $Z_{\Sigma 1}<0.25$, $Z_{\Sigma 1}<0.26$, $Z_{\Sigma 1}<0.27$, $Z_{\Sigma 1}<0.28$, or $Z_{\Sigma 1}<0.29$;
$Z_{\Sigma 2}2<1.2$, such as $Z_{\Sigma 2}<1.11$, $Z_{\Sigma 2}<1.12$, $Z_{\Sigma 2}<1.13$, $Z_{\Sigma 2}<1.14$, $Z_{\Sigma 2}<1.15$, $Z_{\Sigma 2}<1.16$, $Z_{\Sigma 2}<1.17$, $Z_{\Sigma 2}<1.18$, or $Z_{\Sigma 2}<1.19$;
$Z_{\Sigma 3}<-0.39$, such as $Z_{\Sigma 3}<-0.4$, $Z_{\Sigma 3}<-0.41$, $Z_{\Sigma 3}<-0.42$, $Z_{\Sigma 3}<-0.43$, $Z_{\Sigma 3}<-0.45$, $Z_{\Sigma 3}<-0.46$, $Z_{\Sigma 3}<-0.47$, or $Z_{\Sigma 3}<-0.48$;
$Z_{\Sigma 4}<0.43$, such as $Z_{\Sigma 4}<0.34$, $Z_{\Sigma 4}<0.35$, $Z_{\Sigma 4}<0.36$, $Z_{\Sigma 4}<0.37$, $Z_{\Sigma 4}<0.38$, $Z_{\Sigma 4}<0.39$, $Z_{\Sigma 4}<0.4$, $Z_{\Sigma 4}<0.41$, or $Z_{\Sigma 4}<0.42$;
$Z_{\Sigma 5}<1.05$ and $Z_{\Sigma 5}>0.22$, such as $Z_{\Sigma 5}<1.04$ and $Z_{\Sigma 5}>0.21$, $Z_{\Sigma 5}<1.03$ and $Z_{\Sigma 5}>0.20$, $Z_{\Sigma 5}<1.02$ and $Z_{\Sigma 5}>0.19$, $Z_{\Sigma 5}<1.01$ and $Z_{\Sigma 5}>0.18$, $Z_{\Sigma 5}<1.00$ and $Z_{\Sigma 5}>0.17$, $Z_{\Sigma 5}<0.99$ and $Z_{\Sigma 5}>0.16$, $Z_{\Sigma 5}<0.98$ and $Z_{\Sigma 5}>0.15$, $Z_{\Sigma 5}<0.97$ and $Z_{\Sigma 5}>0.14$, or $Z_{\Sigma 5}<0.96$ and $Z_{\Sigma 5}>0.13$.

Additionally, any conservative variant of the sequence of a CPP found, designed and/or verified by a method according to the present invention, and any cell membrane penetrating analogues of a CPP found, designed and/or verified by a method according to the present invention, is by virtue of its functional relationship to said CPP considered to be inside the scope of the present invention.

A conservative variant of a sequence is in the present context defined as an amino acid sequence which is conserved at least 75%, when comparing variants of the same amino acid sequence between different species.

Furthermore, any amino acid sequence being at least 70% identical, such as being at least 72%, 75%, 77%, 80%, 82%, 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical with the amino acid sequence of a CPP, characterised by having a Z_Σ bulk property value essentially consisting of individual average interval values, wherein $Z_{\Sigma 1}$<0.2; $Z_{\Sigma 2}$<1.1; $Z_{\Sigma 3}$<-0.49; $Z_{\Sigma 4}$<0.33; and $Z_{\Sigma 5}$<0.95 and $Z_{\Sigma 5}$>0.12, found, designed and/or verified by a method according to the present invention, is also considered to be inside the scope of the present invention.

By a polypeptide having an amino acid sequence at least, for example, 95% identical to a reference amino acid sequence, is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the amino acid sequence may include up to 5 point mutations per each 100 amino acids of the reference amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence: up to 5% of the amino acids in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acids in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among amino acids in the reference sequence or in one or more contiguous groups within the reference sequence.

In the present invention a local algorithm program is best suited to determine identity. Local algorithm programs, such as (Smith-Waterman) compare a subsequence in one sequence with a subsequence in a second sequence, and find the combination of subsequences and the alignment of those subsequences, which yields the highest overall similarity score. Internal gaps, if allowed, are penalized. Local algorithms work well for comparing two multidomain proteins which have a single domain, or just a binding site in common.

Methods to determine identity and similarity are codified in publicly available programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acid Research 12 (1):387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, S.F., et al., J.Molec.Biol.215:403-410(1990)). The BLASTX program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S.F., et al., NCBI NLM NIH Bethesda, Md. 20894; Altschul, S.F., et al., J.Molec.Biol.215:403-410(1990)). Each sequence analysis program has a default scoring matrix and a default gap penalties. In general, a molecular biologist would be expected to use the default settings established by the software program used.

In the present context, an amino acid is any organic compound containing an amino ($-NH_2$) and a carboxyl (-COOH) group. Amino acids can be in either L- or D- form. There are at present 22 known coded α-amino acids from which proteins are synthesized during

ribosomal translation of mRNA. Additionally, a vast number of non-coded amino acids are constantly emerging, of which 56 examples are given in table 1A. Both coded and non-coded amino acids can of course be part of the amino acid sequences, peptide fragments, peptides, proteins and/or polypeptides included in the present invention.

Amino acid sequence is in the present context the precisely defined linear order of amino acids (including both coded and/or non-coded amino acids) in a peptide fragment, peptide, protein or polypeptide.

In prior art, reporter groups of different characters have been coupled to a putative CPP in order to estimate its cellular translocation and efficiency. The most popular tags have been biotin and different fluorophores, e.g. fluorescein, aminobenzoic acid, and rhodamines. Introduction of these tags into the peptide, however, have often had to be performed manually and the respective derivatives increased the cost of the synthesis remarkably. Moreover, using fluorescence microscopy for detecting said tags intracellularily required several steps including changes of cell medium and washing cells with buffer before visualisation. Even worse, biotin e.g. could only be detected by using an indirect immunofluorescence, which included further steps of fixation, permeabilisation and blocking.

The present invention for the first time discloses a method for verifying the cell-penetrating capacity of a novel CPP and/or a known but improved CPP, which is efficient, fast and reliable, for screening the cellular uptake of a broad variety of CPPs *in vitro* and *in vivo*. The present invention thus relates to a method, wherein the cell-penetrating capacity of a peptide and/or peptide fragment is verified by detecting a change in the membrane potential and/or membrane properties of a cell, monitored as the cellular uptake rate of a membrane potential sensitive substance, such as a dye, a receptor, or an effector, into said cell after exposure to said peptide and/or peptide fragment.

An illustrative example of said method is described in example 10.

$DiBAC_4(3)$ (bis-(1,3-dibutylbarbituric acid)trimethine oxonol) has been widely used for estimation of changes of the membrane potential in response to different stimuli. The fluorescence intensity of $DiBAC_4(3)$ is known to increase three-fold upon the binding/internalisation to the cells. Depolarisation of cells is known to increase the uptake of $DiBAC_4(3)$ and thereby also the fluorescence intensity, while the increase of the membrane potential leads to the decrease in the fluorescence intensity.

It has been generally accepted that cell-penetrating peptides may directly interact with lipid components of the plasma membrane. Insertion of a CPP into the plasma membrane should thus in principle modify the properties (fluidity, permeability etc.) of the membrane and transport through it. Further, the cellular uptake of a CPP could well be dependent on the membrane potentials of cells and therefore could possibly also modify them. Thus, the present inventors in example 10 assessed whether known cell-penetrating peptides

influence the cellular uptake and equilibrium of membrane potential sensitive dye $DiBAC_4(3)$.

Penetratin, transportan and their cell penetrating analogues led to a remarkable increase in $DiBAC_4(3)$ cellular fluorescence, whereas the respective inactive analogues had no impact on the fluorescence intensity, allowing distinction between the penetrating and non-penetrating peptides. Furthermore, as most known CPPs show a tendency to accumulate in membranous structures of cells, the change of amount of fluorescence of $DiBAC_4(3)$ in the cells in the described method monitors both a change of membrane potential, as well as changes of membrane properties.

The term "cell-penetrating capacity" of a peptide will henceforth be used synonymously to its capability to translocate across the plasma membrane into either cytoplasmic and/or nuclear compartments of eukaryotic and/or prokaryotic cells, seemingly energy-independently. Additionally, the term "cell-penetrating capacity" of a peptide can in some aspects of the invention also be used synonymously to indicate transcellular or transmembrane transport, and thus also stand for e.g. the capability to translocate across an epithelial membrane, such as across the epithelium in the intestinal/buccal system, the mucosa in the mouth, lung, anus or nose, or the blood brain barrier of a mammal.

A detected and verified peptide, displaying cellular penetration capacity according to the present invention is in the present context defined as a "cell-penetrating peptide (CPP)" and can e.g. be used for intracellular delivery of macromolecules, such as polypeptides and/or oligonucleotides with molecular weights several times greater than its own.

Cellular delivery using a cell-penetrating peptide and/or a non-peptide analogue thereof is non-invasive, energy-independent, efficient for a broad range of cell types and/or a broad variety of cargo, applicable to cells en masse, non-saturable, and/or receptor independent.

CPPs detected and/or verified by a method disclosed in the present application will be useful for the transport of hydrophilic macromolecules into the cytoplasmic and nuclear compartments of a living cell and/or microorganism, without permanently disrupting the plasma membrane, as well as to deliver hydrophilic macromolecules across the blood-brain barrier, permitting e.g. the intracellular transport of conjugated oligopeptides and oligonucleotides.

Thus, a cell-penetrating peptide and/or a non-peptide analogue thereof might in the present context be used as a delivery vector for any pharmacologically interesting substance, such as a peptide, polypeptide, protein, small molecular substance, drug, mononucleotide, oligonucleotide, polynucleotide, antisense molecule, double stranded as well as single stranded DNA, RNA and/or any artificial or partly artificial nucleic acid, e.g. PNA, as well as as a research tool for delivering e.g. tags and markers and/or for changing membrane potentials and/or properties.

A CPP found or designed according to the present invention can therefore be of use as a vector for the delivery of a hydrophilic biomolecule and/or drug into cytoplasmic and nuclear compartments of a cell and/or a tissue, both *in vivo* and *in vitro*.

When covalently linked with a cargo, including any peptide, polypeptide, protein, small molecular substance, drug, polypeptide and oligonucleotide, with many times its own molecular mass, a CPP might still be able to translocate.

What is more, a CPP can in itself display intra and/or extracellular effector activity, thus function as a cell penetrating functional protein-mimicking peptide.

A cell penetrating functional protein-mimicking peptide is a peptide that will be internalised into the host cell and once inside the host cell will display a mimicking activity of either the original protein or peptide that it has been generated from, or a protein or peptide of choice to that it has been designed to mimic. A cell penetrating functional protein-mimicking peptide is thus defined as a CPP that in itself has effector activity and that will activate an internal and/or external signalling pathway and/or cascade, resembling the activated functional protein that it is derived from. It is therefore characterised as having both cellular penetrating capability and effector and/or functional protein-mimicking activity.

A cellular effector can herein be either an intracellular and/or extracellular effector and is in the present context defined as a structure that produces a cellular effect, such as a contraction, secretion, electrical impulse, or activation of an intracellular and/or extracellular signaling cascade, or that induces the up regulation of a cellular level of an mRNA and/or a protein, in response to a stimulation by said effector. A typical effector is in the present context selected from the group consisting of a metabolite, an antagonist, an agonist, a receptor ligand, a receptor coupled protein, an activated receptor, an enzyme inhibitor, activator and/or stimulator, a kinase, a phosphatase, an enhancer, or a silencer, a transporter and/or a transmitter, a hormone, a channel, an ion, a prion, or a viral protein.

A typical CPP detected and verified by a method according to the present invention can be derived from a broad variety of proteins and/or peptides. In one embodiment, said protein and/or peptide is a transmembrane protein, and in yet another embodiment, it can as well be a non-membrane associated protein.

Most preferably, though, the CPP detected and verified by a method according to the present invention, is derived from a transmembraneous protein, such as a membrane associated receptor or from a receptor agonist and/or antagonist. A cell penetrating functional protein-mimicking peptide is in this embodiment most preferably derived from a membrane associated receptor or designed to closely resemble a membrane associated receptor or at least a fragment of a membrane associated receptor. More preferably still, the CPP is derived from an intracellular part or loop of said membrane associated receptor.

As found for many G-protein coupled receptors, some synthetic peptides, derived from their intracellular loops, influence receptor-G-protein interactions in membrane preparation. Thus in a most preferred embodiment of the present invention, said cell penetrating functional protein-mimicking peptide is derived from a mammalian receptor, such as a receptor belonging to a protein family which can be classified based on its member's structure and their function and comprises channel receptors, tyrosine kinase receptors, guanylate cyclase receptors, serine/threonine kinase receptors, cytokine receptors, and receptors coupled to guanosine triphosphate (GTP)-binding proteins (G protein-coupled receptors: GPCRs).

GPCRs are in the present context defined as having seven transmembrane domains, three extracellular loops (e_1, e_2, e_3) and four intracellular loops (i_1, i_2, i_3, i_4). A cell penetrating functional protein-mimicking peptide is thus preferably derived or resembles a fragment of any of the intracellular or extracellular loops of said receptors.

In an even more preferred embodiment, said cell penetrating functional protein-mimicking peptide is derived from the group consisting of the GLP-1 receptor, AT1A receptor, CGRP receptor, and Dopamine receptor.

Nonetheless, a cell penetrating functional protein-mimicking peptide can equally well be derived from or resemble any other cellular effector, such as an enzyme, channel, hormone, transcription factor, receptor agonist or antagonist, transporter, or ligand, and can e.g. be derived from or resemble platelet-activating factor (PAF), CGRP, thyroid-stimulating hormone (TSH), luteinizing hormone (LH), or follicle-stimulating hormone (FSH).

As described above, a CPP can stem from a receptor activating ligand, or an internal loop, or a transmembraneous loop of a receptor and thus have internal activating properties, but can also be solely transporting cargo across a membrane. Thus, in the present context, CPPs are divided into two classes:

a. functional protein-mimicking CPPs
b. cargo-transporting CPPs

None withstanding, a CPP belonging to group a) will of course in most cases also be capable and useful for cargo-transport.

The following will exemplify a variety of different CPPs detected and verified with a method according to the present application. Given the magnitude of potential CPPs that can for the first time be detected with the different methods disclosed in the present application, naturally, the selection of specific and novel CPPs and improved usage of known CPPs given herein is purely meant to be illustrative and by no means exhaustive.

Examples for CPPs, detected and verified by a method according to the present application, are given in the experimental section, and are also solely intended to be illustrative and by no means exhaustive.

References mentioned in the present application are considered to be incorporated.

Receptor derived CPPs:

One preferred embodiment of the present invention comprises a novel synthesized peptide: GOP, derived from the glucagon like peptide 1 receptor, GLP-1 receptor (as described in detail in example 5). The novel CPP acts as a potent mimicker of action of the GLP-1 receptor, i.e. it increases insulin release, when incubated with rat and human pancreatic islets. Further, the peptide and cell membrane penetrating analogues thereof are able to localize intracellularly, when incubated with cells and act as mimics of an agonist of GLP-1 receptor protein action. Thus, they are potential powerful candidates for treatment of non-insulin dependent diabetes mellitus, NIDDM, and generally for treating both diabetes type I and II.

The above described novel transport peptides, or any other receptor derived CPP, are universal transport peptides, functional protein-mimicking CPPs, as well as cargo-transporting CPPs, and can be used for cellular delivery of a variety of cellular effectors, e.g. general modifiers of intracellular and/or extracellular metabolic and signaling mechanisms, such as peptides, proteins, oligonucleotides and polynucleotides and/or for the delivery of antibiotics and antiviral agents into cells and microorganisms.

Consequently, one specific aspect of the present invention is directed to a peptide selected from the group consisting of peptides having the amino acid sequence IVIAKLKA (GOP), conservative variants of the sequence and cell membrane penetrating analogues thereof.

The cell penetrating analogues of peptide GOP (being in example 5 derived from rat GLP-1 receptor) may e.g. be corresponding peptides from other mammalian species or individual variants from the same species, and may thus have amino acid extensions, deletions or substitutions in relation to the amino acid sequence of peptide GOP, as long as they have cell penetrating properties/capability. A representative example of this type of cell-penetrating peptides, held in the scope of the present invention, is IVIAKLKANLMCKTCRLAK-amide (M 569). Cell penetrating properties of said analogues of GOP can easily be tested by a variety of standard methods, well known to the skilled artisan, or as illustrated in example 2.

Another aspect of the invention is directed to the above disclosed CPP of the invention for use as a medicament, in particular a medicament for the treatment of insulin deficiency in non-insulin dependent diabetes mellitus. Consequently, any other receptor-derived CPP can of course be used as a medicament for treating any disease or abnormal condition correlated to the receptor that said CPP is derived from. Typically, such diseases are selected from the group consisting of metabolic diseases or disorders, such as diabetes

type I and type II, neurological diseases, such as Alzheimer's Disease, Huntington-Chorea, Parkinson's Disease, or epilepsy, taste and smell disorders, a disease with oncogenic properties, ulcer, addiction and abuse disorders, infectious diseases, inflammations, pain, immunological diseases or disorders, such as asthma and allergy, immunological suppression, immunological hyper function, or autoimmune diseases.

In a most preferred embodiment, wherein said CPP is derived from a G protein-coupled receptor, or designed to mimic a G protein-coupled receptor, said CPP is used for the manufacture of a pharmaceutical composition for the treatment of a disease with oncogenic properties, including toxic thyroid hyperplasia (mutated thyroid-stimulating hormone (TSH) receptor), retinis pigmentosa (mutated rhodopsin), precocious puberty (mutated luteinizing hormone (LH) receptor), hypocalcaemia (mutated Ca^{2+} receptor) and Jansen metaphyseal chondrodysplasia (mutated parathyroid hormone and parathyroid hormone-related peptides (PTH/PTHrP) receptors). Furthermore, said composition can also be used for the treatment of a pathology associated with inactivation of GPCRs such as X-linked nephrogenic diabetes insipidus (vasopressin V2 receptor), familial glucocorticoid deficiency (adrenal corticoid hormone (ACTH) receptor), bleeding disorder (thromboxane A_2 receptor), male pseudohermaphroditism (LH receptor), familial hypocalciuric hypercalcaemia, neonatal hyperparathyroidism (Ca^{2+} receptor) or Hirschprung disease (endothelin B receptor).

Still another aspect of the invention is directed to a method of treating insulin deficiency in a patient having non-insulin dependent diabetes mellitus, comprising the steps of administering to said patient an insulin release increasing amount of a peptide according to the invention, or a pharmaceutical composition according to the invention. The insulin release increasing amount will be recommended by the attending physician with guidance from the manufacturer and the response from the patient.

Yet another aspect of the invention is directed to a pharmaceutical composition comprising, as an active ingredient, a peptide according to the invention, together with a pharmaceutically acceptable vehicle. The vehicle is selected by the manufacturer based on the desired route of administration, and examples of suitable vehicles can be found in the US or European pharmacopoeia.

In yet another highly preferred embodiment, the present invention relates to a novel vasoconstrictor, more precisely to a synthetic peptide derived from the intracellular C-terminus of angiotensin 1A receptor. The peptide is a functional protein-mimicking CPP and promotes contraction of heart coronary blood vessels.

The CPP related to herein is derived from the AT1A receptor, comprising a peptide corresponding to at least one fragment of the C-terminal tail, comprised in the third and/or second intracellular loop of the receptor.

The disclosed selective antagonists of AT1 receptor are potential antihypertensive drugs and are of interest as potential drugs useful in the situations where vasoconstriction is required, e.g. chronical hypotension or migraine.

The present invention thus comprises the synthesized peptide M511, derived from the C-terminal intracellular part of the rat AT1A receptor. M511 is able to translocate into human melanoma cell line Bowes. The effects of M511 and biotinylated M511 were tested with porcine coronary arteries and veins, as well as with human umbilical blood vessels. In all cases, peptides triggered contraction of blood vessels. The sequence to which the M511 peptide corresponds is conserved within the AT1 receptor subfamily, but has low similarity to AT2 type receptor **(Table 2)**.

Table 2. Comparison of C-terminal fragments of rat, mouse and human angiotensin receptors. The sequence of M511 is highlighted, putative transmembrane helixes are underlined and mutations in sequences are in **bold**.

origin	Segments 291 – 330 for AT1 and 307- 346 for AT2 receptors
Rat AT1A	AYFNNCLNPL FYGFLGKKFK KYFLQLLKYI PPKAKSHSSL
Mouse AT1A	AYFNNCLNPL FYGFLGKKFK KYFLQLLKYI PPKAKSHSSL
Human AT1A	AYFNNCLNPL FYGFLGKKFK RYFLQLLKYI PPKAKSHSNL
Rat AT1B	AYFNNCLNPL FYGFLGKKFK RYFLQLLKYI PPKARSHAGL
Mouse AT1B	AYFNNCLNPL FYGFLGKKFK RYFLQLLKYI PPKARSHAGL
Human AT1B	AYFNNCLNPL FYGFLGKKFK KDILQLLKYI PPKAKSHSNL
Rat AT2	GFTNSCVNPF LYCFVGNRFQ QKLRSVFRVP ITWLQGKRET
Mouse AT2	GFTNSCVNPF LYCFVGNRFQ QKLRSVFRVP ITWLQGKRET
Human AT2	GFTNSCVNPF LYCFVGNRFQ QKLRSVFRVP ITWLQGKRES

Although the inventors have not demonstrated its selectivity to AT1 mediated signal transduction, they show that the peptide activates specifically the same type of G-proteins as agonist activated AT1A receptor. Scrambled M511 **(Table 3)** were prepared and tested as a control, with no success in blood vessel contraction.

Table 3A. Sequences of penetratin, M511 and scrambled M511.

Name	Sequence
Penetratin	RQIKIWFQNRRMKWKK
M511	FLGKKFKKYFLQLLK
ScrM511	KGKFQLYLKLKFKFL

Table 3B. novel analogues of M511
(Cit-citrulline, Fph- 4-fluoro-phenylalanine)

Name	Sequence/Name
Analogue 1	KKFKKYFL

Analogue 2	KKYFLQLLK
Analogue 3	FKKYFLQLL
Analogue 4	KKFKKYFLQ
Analogue 5	Cit-Cit-Phe-Cit-Cit-Fph-Ile
Analogue 6	Cit-Cit-Fph-Ile-Cit-Ile-Ile-Cit
Analogue 7	Phe-Cit-Cit-Fph-Ile-Cit-Ile-Ile
Analogue 8	Cit-Cit-Phe-Cit-Cit-Fph-Ile-Cit

Unique properties of the novel peptide M511 are e.g. that it penetrates cell membranes by a non-endocytotic mechanism, it induces long-lasting contraction of blood vessel and this contraction is peptide sequence specific. It interacts with G-proteins and mimics agonist activated AT1A receptor.

Thus, one aspect of the invention is directed to a peptide selected from the group consisting of peptides having the amino acid sequence FLGKKFKKYFLQLLK (= M511) and to cell membrane penetrating analogues thereof.

The cell penetrating analogues of the peptide M511 (derived from rat AT1A receptor) may be corresponding peptides from other mammalian species or individual variants from the same species, and thus may have amino acid extensions, deletions or substitutions in relation to the amino acid sequence of the peptide M511, as long as they have cell penetrating properties.

In an additional embodiment of the invention, a CPP as described above is coupled to a cargo. The cargo may be a marker molecule, such as biotin.

Another aspect of the invention is directed to the peptide of the invention for use as a vasoconstrictor.

Yet another aspect of the invention is directed to a pharmaceutical composition comprising, as an active ingredient, a peptide according to the invention, together with a pharmaceutically acceptable vehicle. The vehicle is selected by the manufacturer based on the desired route of administration, and examples of suitable vehicles can be found in the US or European pharmacopoeia.

Still another aspect of the invention is directed to a method of inducing contraction of blood vessels in an individual comprising the steps of administering to said individual a vasoconstricting amount of a peptide according to the invention, or a pharmaceutical composition according to the invention.

Transmembrane-protein derived CPPs:

In another, equally preferred embodiment, a CPP related to in the present context can stem from any other transmembrane peptide, and is by no means limited to being derived from a receptor. As disclosed in example 6, one embodiment of the present invention thus relates to a CPP derived from mouse PrpC (1-28): MANLG YWLLA LFVTM WTDVG LCKKR PKP, human PrpC(1-28): MANLG CWMLV LFVAT WSDLG LCKKR PKP, or bovine PrpC (1-30): MVKSK IGSWI LVLFV AMWSD VGLCK KRPKP.

As disclosed in example 7, even amyloid precursor protein (APP) and presenilin-1 (PS-1) have cell-penetrating sequences and are consequently included as sources for a CPP derived from their amino acid sequence, according to a method described in the present application.

Detected potential CPPs

In the present context, cell-penetrating peptides can be derived from both random peptide sequences, and from naturally occurring proteins.

A cell-penetrating peptide and/or a non-peptide analogue thereof detected by a method according to the present invention is preferably selected from a 8 to 50 amino acid residues long peptide, such as a 8 to 30 amino acid residues long peptide, or a 14 to 30 amino acid residues long peptide, or a 16 to 20 amino acid residues long peptide.

In one embodiment of the invention, a cell-penetrating peptide is selected from a 12 to 50 amino acid residues long peptide or a fragment of a peptide of one of the amino acid sequences as listed in the accompanying sequence listing as SEQ ID NO. 1-150.

In another embodiment of the invention, a cell-penetrating peptide is selected from a 12 amino acid residues long peptide or a fragment of a peptide of one of the amino acid sequences as listed in the accompanying sequence listing as SEQ ID NO. 151-2684.

In yet another embodiment of the invention, a cell-penetrating peptide is selected from a 16 amino acid residues long peptide or a fragment of a peptide of one of the amino acid sequences as listed in the accompanying sequence listing as SEQ ID NO. 2685-6233.

Cargo

As described previously, a CPP can be coupled to a cargo or carrier. The cargo may be selected from the group consisting of any pharmacologically interesting substance, such as a peptide, polypeptide, protein, small molecular substance, drug, mononucleotide, oligonucleotide, polynucleotide, antisense molecule, double stranded as well as single stranded DNA, RNA and/or any artificial or partly artificial nucleic acid, e.g. PNA, a small molecule, saccharide, plasmid, antibiotic substance, cytotoxic and/or antiviral agent. Furthermore, the transport of cargo can be useful as a research tool for delivering e.g.

tags and markers as well as for changing membrane potentials and/or properties, the cargo may e.g. be a marker molecule, such as biotin.

With respect to the intended transport of cargo across the blood brain barrier, both intracellular and extracellular substances are equally preferred cargo.

Naturally, not every CPP will be equally qualified for transporting any and each cargo, such as has e.g. been shown for Tat and Penetratin, not being optimal for transporting highly negative charged cargo, such as DNA. Thus, the selection of most optimal CPP of choice for transporting a certain cargo will have to be estimated and verified by the person skilled in the art, and will be highly dependent on the nature of the specific cargo and the target cell/tissue.

In a preferred embodiment of the invention, the cell-penetrating peptide is coupled by a S-S bridge to said cargo. Naturally, there is a broad variety of methods for coupling a cargo to a CPP, selected individually depending on the nature of CPP, cargo and intended use. A mode for coupling can be selected from the group consisting of biotin-avidin binding, covalent and non-covalent binding, esters, amides, and antibody bindings.

In some embodiments, a labile binding is preferred, in other embodiments, a stabile binding is elementary, such as in the use of a CPP according to the present invention for use in transport of medical substances, due to the necessary storage of said pharmaceutical compositions before use.

Improvement of a known cellular penetration method

Additionally, a CPP discovered by a method according to the present invention can also be used for the improvement of a known cellular penetration method, such as for the improvement of gene delivery *in vivo,* comprising transfection, microinjection, transduction or electroporation.

During the past 40 years, DNA delivery, especially via the non viral route (i.e., transfection), has become a powerful research tool for elucidating gene function and regulation. Nonviral gene delivery systems generally exhibit a superior safety compared to viruses, which are more commonly used especially in clinical trials, however, their relatively low efficiency of transgene expression is a major obstacle (Ma, H. & Diamond, S.L. Nonviral gene therapy and its delivery systems. *Curr Pharm Biotechnol* **2**, 1-17. (2001)). The efficiency of DNA delivery is dependent on several steps: adsorption of transfection complex to the cellular surface, uptake by the cell, endosomal release, nuclear translocation and expression of the gene.

Nonviral transfection methods

Nonviral transfection reagents available today are mainly working in three ways: increasing the uptake of the plasmid across the plasma membrane, destabilizing the endosomal membrane and enhancing nuclear uptake. The main transfection protocols and

reagents include: 1) calcium phosphate precipitation; 2) cationic polymers as DEAE-dextran, polylysine and polyethyleneimine (PEI) (Garnett, M.C. Gene-delivery systems using cationic polymers. *Crit Rev Ther Drug Carrier Syst* **16**, 147-207 (1999)); 3) physical methods like microinjection and electroporation, DNA gun and similar (Somiari, S. *et al.* Theory and in vivo application of electroporative gene delivery. *Mol Ther* **2**, 178-87. (2000)); and 4) liposomal vectors like cationic and anionic liposomes (Lee, R.J. & Huang, L. Lipidic vector systems for gene transfer. *Crit. Rev. Therap. Drug Carrier Syst.* **14**, 173-206 (1997)). Many of these methods and transfection reagents are working well *in vitro* and for ex vivo transfections but are less suitable for *in vivo* gene transfer (Ma, H. & Diamond, S.L. Nonviral gene therapy and its delivery systems. *Curr Pharm Biotechnol* **2**, 1-17. (2001)). In general, methods with high delivery efficiency are also toxic for the cells. The only exception is microinjection, which is both effective in delivery and non-toxic, but unfortunately can not be used en masse (Luo, D. & Saltzman, W.M. Enhancement of transfection by physical concentration of DNA at the cell surface. *Nat Biotechnol* **18**, 893-5). Old chemical reagents and methods like DEAE-dextran and calcium phosphate precipitation are simple, effective and still widely used but both suffer of cytotoxicity and are difficult to apply in vivo. Also, they lack cell specific targeting and the structure of DNA-lipid complexes are poorly understood.

For introduction of DNA into cells, favourite methods have been complexing with different compounds. This approach allows easy preparation of transforming agent and therefore quick modification of DNA construct and transformation conditions. Primary role of complexing agents is neutralization of the negative charge of phosphate groups in the DNA backbone and condensing the large DNA molecule. An average DNA molecule used for delivery of foreign DNA has to be at least 3000 base pair long to be propagated in bacterial cells during preparation. Most of modern plasmids for mammalian cell expression are 4500 to 5000 bp long i.e. have Mw 1.500.000. After neutralization of negative charge and packing into tight particles DNA molecules are taken up by cells via endocytotic pathways. Classical transfection methods/agents have been modified in many ways attempting to prevent or neutralize degradation pathway activation in response to endocytosis.

Ca-phosphate transfection method remains still the most popular and widely used. The main reason for the popularity is very low cost. However, the method is extremely cell type specific and toxic for many cell types including neuronal and primary cells. Many attempts have been made to include DNA into liposome-like structures. Other methods relay on complexing of DNA with polymeric molecules that bind to DNA. A major problem with all those approaches have been toxicity to cells.

Polyplex technique

Another approach that has gained a lot of prominence in last years, is the use of transfection systems based on the principle of condensing DNA with polycations. According to renewed nomenclatura, this technique is referred to as polyplex (Felgner, P.L. *et al.* Nomenclature for synthetic gene delivery systems. *Hum Gene Ther* **8**, 511-2. (1997)). Polyplexes are more effective than lipid based vectors and also, in most cases, less toxic

(Gebhart, C.L. & Kabanov, A.V. Evaluation of polyplexes as gene transfer agents. *J Control Release* **73**, 401-16. (2001)). One of the cheapest, very effective and most widely used polycation is polyethyleneimine (PEI) (Boussif, O. *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* **92**, 7297-301. (1995),Abdallah, B. *et al.* A powerful nonviral vector for in vivo gene transfer into the adult mammalian brain: polyethylenimine. *Hum Gene Ther* **7**, 1947-54. (1996), Schatzlein, A.G. Non-viral vectors in cancer gene therapy: principles and progress. *Anticancer Drugs* **12**, 275-304. (2001)). Since it has a high positive net charge, it neutralises negative charges of dsDNA and also condenses DNA. Compact PEI/DNA globules internalise into cells mostly by endocytosis (Remy-Kristensen, A., Clamme, J.P., Vuilleumier, C., Kuhry, J.G. & Mely, Y. Role of endocytosis in the transfection of L929 fibroblasts by polyethylenimine/DNA complexes. *Biochim Biophys Acta* **1514**, 21-32. (2001)). These complexes also promote transfection by preventing degradation of DNA by lysosomal enzymes and by enhancing the release of DNA from the endocytic vesicles (Gebhart and Kabanov 2001). Unfortunately, though, at concentrations successfully used *in vitro*, the polycations are still too toxic for systemic *in vivo* use.

The approach described in the present application discloses a principally new way of transporting large DNA molecules across a cell membrane. Instead of relying on cell-activity-dependent endocytosis, an active transport of plasmids is proposed, using the capacity of cell-penetrating peptides (CPP) to carry cargoes into cells. While CPPs are performing the transport action, a second component neutralises phosphate groups and condenses (packs) the large DNA molecule. Further, packing agents that mask the phosphates have always had an additional function as proton buffers. Binding of protons neutralises lysosomes and inhibits many degradation pathway enzymes. Also, the escaping of the degradation pathways by active transport over the cell membrane dramatically reduces the amount of phosphate neutralising/packing agent necessary, and therefore lowers its toxic effects.

As described in example 9, the invention thus also relates to an improved polyplex mediated gene delivery method, wherein a cell-penetrating peptide and/or a non-peptide analogue thereof is conjugated either to a reporter gene or to a transfection reagent, such as e.g. polyethylene amine. In both cases enhanced expression of reporter proteins GFP and luciferase, are observed.

Table 4 Comparison of transfection methods with PEI-TP10 methods

Method	Transfection efficiency	Toxicity		Remarks
		in vitro	*in vivo*	
Viruses				
adenovirus	+++	low	variable	immunogenic
Retrovirus	+++	low	low	revertant risk
Lentiviruses	+++	low	unknown	
Microinjection	+++	low	not applicable	
Electroporation	++(+)	high	high	
$Ca_3(PO_4)_2$ precipitation	++	high	high	
Lipofection				
cationic liposomes	++	medium	high	
anionic liposomes	+(+)	medium	high	
Polycations				
DEAE- dextran	+(+)	medium	medium	
Polylysine, Polyornithine	+(+)	low	medium	
Loligomers	+(+)	medium	medium	
dendrimers	++	medium	medium	
polyethyleneimine	++	medium	medium	
Trasferrin-PEI	++	low	medium	cell selective
Polyethylene glycosylated PEI	++	low	medium	cell selective
PNA-NLS+PEI	++(+)	low	unknown	low PEI doses
present method	++(+)	low	low	low PEI doses

Consequently, the present invention in particular relates to a vector for (non-viral) cell transfection, comprising a) a nucleic acid component, b) a polycation conjugate, and c) a cell-penetrating peptide and/or a non-peptide analogue thereof, which is able to enhance the average rate of transfection per cell at identical transfection conditions by a factor of at least 2, such as by a factor of at least 5, 10, or 15, compared to a vector comprising only components a) and b), or only a) and c).

Also envisioned herein is a vector as described above, for usage in a transient transfection and/or a stable transfection of a cell *in vivo* and/or *in vitro*, for transfecting a mammalian cell such as a cell selected from the group consisting of human, rodent, pig, cow.

A vector as described above will typically comprise DNA as oligonucleotide and/or polynucleotide and said polycation conjugate will be polyethyleneimine (PEI), polyornithine, polylysine, polyamines, dendrimers, spermidine, DEAE-dextran, patricine, transferrin-PEI, polyethylene glycosylated PEI, or loligomers.

Consequently, the present invention also relates to a method for *in vivo* transfecting a cell in a host tissue with a nucleic acid, comprising introducing a vector according to the present invention, e.g. as illustrated in example 9, for *in vivo* transfecting a cell in a host tissue and/or an isolated cell with a nucleic acid.

Cell selective CPPs

In yet a further embodiment of the present invention, a cell-penetrating peptide and/or a non-peptide analogue thereof is provided that will enter selectively into a certain cell type, or that transports a cargo that will only be activated in a certain cell type.

The inventors show that different CPPs are internalised by specific cell lines, such as human melanoma cell line Bowes and others, with significantly different efficacy and rate of uptake, sometimes more than two-fold. This is a prerequisite to define CPPs that are internalised with different efficacy to different cell lines and tissues. Hence, an important embodiment of the present invention is a method for the development of selective CPPs (selCPPs) characterised e.g. by testing all available CPPs for cellular uptake in cell lines, cells, or tissues into which a selective transport is required. On the other hand, certain selective methods might be employed to artificially enhance the cell selectivity of a CPP of choice for a certain target cell or target cell population, which will be described in detail below.

As an example, cancer cells expose many cell surface antigens and/or proteins. Usually, tumour cells do not express cell surface markers that are unique but rather over-express common receptors/markers. The signalling through these over-expressed markers and/or over-amplifications of the intracellular and/or extracellular signals is thought to be one of the mechanisms for the loss of control of the cellular machinery over the cell cycle. Thus, in a specific embodiment of the present invention, an over-expressed cell surface protein is applied as target for CPP addressing.

In one embodiment of the invention, a cell selective CPP (selCPP) is envisioned that comprises an antigen/protein raised against a cellular marker, selected from the group consisting of channel receptors, tyrosine kinase receptors (e.g. EGF, IGF), guanylate cyclase receptors, serine/threonine kinase receptors, cytokine receptors, receptors coupled to guanosine triphosphate (GTP)-binding proteins (G protein-coupled receptors: GPCRs), glycosphingolipids, CD44, neuropeptide receptors, e.g. neurotensin receptors, galanin, and substance P receptors.

Generally, a cell selective CPP (selCPP) will of course be extremely useful in the targeted transport of any kind of drug or pharmaceutical substance to a variety of specific

eukaryotic and/or prokaryotic cellular targets. A cell selective transport of such cargo is e.g. envisioned for an improved treatment or prevention of infectious diseases, such as diseases caused by a viral, bacterial or parasital infection.

In order to avoid non-specific internalisation of CPPs before finding a target cell in vivo, the invention further relates to a selCPP being designed so that the active structure of the CPPs is disrupted; only the binding event of a peptide part of said selCPP to a specific receptor/marker releases the CPP from conformational discrimination, i.e., the prodrug strategy is used.

Cell type targeted CPPs can further be modified by a non-covalent intermolecular interaction with the part of a receptor targeting sequence. After binding to the receptor, the CPP is herein displaced by a receptor and the CPP will internalise, Fig.1 and Fig.2. Receptor internalisation is relatively slow, as compared to the CPP translocation.

An especially preferred embodiment of the present invention thus relates to a cell selective delivery system for a cytostatic agent, comprising a) a protease consensus site for a protease specifically overexpressed in a target cell, b) a cell-penetrating peptide and/or a non-peptide analogue thereof, and c) a cytostatic agent, wherein said cell selective delivery system additionally comprises an inactivation sequence repressing the cellular penetration capacity of said cell-penetrating peptide, and which is cleaved by said protease specifically overexpressed in the target cell upon introducing said cell selective delivery system in the near vicinity of said target cell.

Matrix metallo proteases (MMPs) are Zn^{2+} metallo endopeptidases. The family contains both membrane bound and secreted members of which both catalyse the breakdown of proteins located either on the cell's plasma membrane or within the extracellular matrix (ECM) (M.D. Sternlicht and Z. Werb " How matrix metallo proteinases regulate cell behavior" Annual Review of Cell and Developmental Biology, 17:463-516, 2001). MMPs have been linked to the invasive and metastatic behaviour of a wide variety of malignancies, and these enzymes are generally overexpressed in a variety of tumors (M.D. Sternlicht and Z. Werb " How matrix metallo proteinases regulate cell behavior" Annual Review of Cell and Developmental Biology, 17:463-516; D.V. Rozanov et al., " Mutation analysis of membrane type-1 metalloproteinase (MT1-MMP)", Journal of Biological Chemistry (JBC), 276:25705-14, July 13, 2001). Membrane type MMPs (MT-MMP), such as MMP-MT1 have been strongly implicated in oncogenesis. These enzymes localise to the invasive fronts. The soluble MMPs 1-3, 9 and 14 have also been implicated as agonists of tumorigenesis (Smith, L.E., Parks, K.K., Hasegawa, L.S., Eastmond, D.A. & Grosovsky, A.J. Targeted breakage of paracentromeric heterochromatin induces chromosomal instability. *Mutagenesis* **13**, 435-43. (1998)).

Concomitantly, the present invention relates to a method for designing a selCPP, based on three basic functions: 1) selective cleavage (and thereby activation) by MMP-2 or MMP-MT1, 2) cellular penetration by peptides (CPPs) and 3) killing of nearby, preferably tumour

cells or endothelia involved in tumour neovascularisation, by a known cytostatic agent. (see Fig.3 and Fig.4)

The present invention thus comprises a selCPP selected from an amino acid sequence contained in table 5 or table 6, and to a combined cell selective delivery system for a cytostatic agent, comprising an amino acid sequence listed in table 5 or table 6, and a cytostatic agent.

Table 5. selCPPs based on MMP-2 (gelatinase-A) cleavage specificity:

Name	Sequence	MMP site	Penetra-tion	Comment:
YTA-1	YTAIRGIAVLFRCK	SGESLAY-YTA	-/+	localise in plasma membrane
YTA-2	YTAIAWVKAFIRKLRK	SGESLAY-YTA	+++	stain also nuclear membrane Fig.3
YTA-2ps*	SGESLAY-YTAIAWVKAFIRKLRK	SGESLAY-YTA	+	bind the plasma membrane Fig.4

*ps stands for proteinase cleavage site

Table 6. selCPPs based on MMP-MT1 cleavage specificity

Name	Sequence	MMP site
LRSW-1	LRSWVISRSIRKAA	GPLG-LRSW
LRSW-2	LRSWIRRLIKAWKS	GPLG-LRSW
LRSW-3	LRSWRVIIRNGQR	GPLG-LRSW

Consequently, the present invention also relates to a cell selective delivery system for a cytostatic agent, comprising a) a cell-penetrating peptide and/or a non-peptide analogue thereof comprising a protease consensus site for a protease specifically overexpressed in a target cell and c) a cytostatic agent, wherein said cell selective delivery system additionally comprises an inactivation sequence repressing the activity of said cell-penetrating peptide, and which is cleaved by said protease specifically overexpressed in the target cell upon introducing said cell selective delivery system in the near vicinity of said target cell.

In a preferred embodiment of said cell selective delivery system, as described above, said cell-penetrating peptide comprised in said cell selective delivery system enhances the average rate of cellular uptake of said cytostatic agent into said selective cell per cell by a factor of at least 2 compared to the average rate of cellular uptake into said cell of a cell selective delivery system comprising only components a) and c), or to the average rate of cellular uptake of component c) alone of said cell.

In another, equally preferred embodiment of said cell selective delivery system, as described above, said cell-penetrating peptide comprised in said cell selective delivery

system enhances the average rate of cellular uptake of said cytostatic agent into said selective cell per cell by a factor of at least 1.5, such as at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 1000 or 10 000, compared to the average rate of cellular uptake into said cell of a cell selective delivery system comprising only components a) and c), or to the average rate of cellular uptake of component c) alone of said cell.

In another equally preferred embodiment of said cell selective delivery system as described above, said overexpressed protease is a Zn^{2+}metallo endopepdidase selected from the group consisting of MMP-1, MMP-2, and MMP-MT1.

In yet another embodiment, said overexpressed protease is selected from the group consisting of bacterial surface proteases and viral enzymes.

A method is also enclosed in the scope of the present invention for targeting CPPs to particular cells and tissues, i.e. to design and apply selCPPs. A suitable CPP is herein designed with a unique surface marker, which is specific for the designated cell or tissue type, characterised by selecting a CPP from any suitable CPP sequence that is designed by the prediction/selection criteria or found in any other way, selecting peptide X, or address, as an epitope from the suitable cell surface receptor against which a specific monoclonal antibody has been raised against and which recognizes this particular epitope with high affinity, choosing a linker among polypeptides (Gly_n, Pro_n, $GABA_n$, Aha_n, etc.), or suitable organic substances in order to achieve required interactions between Peptide X/antibody and CPP/plasma membrane.

Enscoped in the present application is furthermore an *in vivo* and/or *in vitro* method for stopping cellular proliferation of a specific cellular population and the use of a cell selective delivery system as described herein for *in vivo* and/or *in vitro* stopping cellular proliferation of a specific cellular population.

A cell selective delivery system as described above can of course be used for the manufacture of a pharmaceutical composition for stopping cellular proliferation of a specific cellular population in a mammal and for treating a patient suffering from a medical condition characterised by uncontrolled cellular growth, such as any oncological disorder or disease, or immunological and/or metabolic hyperfunction.

A general aspect of the present invention comprises the use of a cell selective delivery system, or CPP related to in the present invention for the manufacture of a pharmaceutical composition for gene therapy and to the pharmaceutical composition comprising said cell selective delivery system, or CPP.

Another aspect of the invention is directed to a composition comprising a cell-penetrating peptide and/or a non-peptide analogue thereof, or cell selective delivery system according to the invention or resulting from performing any one of the methods according to the

invention, and a compound selected from peptides, oligonucleotides and proteins that are general modifiers of intracellular metabolic or signalling mechanisms, either inhibiting or activating.

Yet another aspect of the invention is directed to the use of a cell-penetrating peptide and/or a non-peptide analogue thereof, or cell selective delivery system according to the invention or resulting from performing any one of the methods according to the invention, for the manufacture of a medicament.

A further aspect of the invention is directed to the use of a composition according to the invention for the manufacture of a medicament.

The different aspects and embodiments of the invention will now be illustrated by the following examples. It should be understood that the invention is not limited to any specifically mentioned details.

ABREVIATIONS

Aβ	beta-amyloid
AD	Alzheimer's disease
APP	Amyloid precursor protein
AT1	angiotensin receptor type AT1
AT1A	angiotensin receptor subtype AT1A
AT1B	angiotensin receptor subtype AT1B
AT2	angiotensin receptor type AT2
BACE	β-site APP-cleaving enzyme
Bio	biotin, biotinylated
BSA	bovine serum albumin
CPP	cell penetrating peptide
CTF	C-terminal fragment
DCC	N,N'-dicyclohexylcarbodiimide
DCM	dichloromethane
DIEA	diisopropylethylamine
DMF	dimethylformamide
DNP	dinitrophenyl
EOFAD	early onset Alzheimer's disease
FITC	5-fluorescein isothiocyanate
Fmoc	9-fluorenylmethoxycarbonyl
GOP	IVIAKLKA-amide
GPCR	G-protein coupled receptor
GTP	guanosine 5'-triphosphate
GTPase	guanosine triphosphatase
GTPγS	guanosine γ-S-5'-triphosphate
HKR	Hepes-Krebbs-Ringer
HOBt	N-hydroxybenzotriazole
HPLC	high performance liquid chromatography
IDE	insulin degrading enzyme
LOAD	late onset Alzheimer's disease
M511	FLGKKFKKYFLQLLK-amide
MBHA	4-methylbenzhydrylamine
NFT	neurofibrillary tangles
NICD	notch intracellular domain
NMP	N-methylpyrrolidone
NTF	N-terminal fragment
PAF	paraformaldehyde
PBS	phosphate buffered saline
PNA	peptide nucleic acid
PS	presenilin

RNA	ribonucleic acid
SAPP	secretory APP
TACE	tumour necrosis factor alpha converting enzyme
t-Boc	*tert*-butyloxycarbonyl
TBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TFA	trifluoroacetic acid
TFA	trifluoroacetic acid
TFMSA	trifluoromethanesulphonic acid

LEGENDS TO FIGURES

Fig. 1. Scheme of addressing selCPP by application of interaction with single transmembrane (A) or 7-transmembrane (B) receptor.

Fig. 2. Intramolecularly constrained selCPP looses its constrain upon recognition event by specific receptor and the internalisation takes place.

Fig. 3. Schematic structure of chimeric selCPP.

Fig. 4. Incubation of non-covalent selCPP-AB complex with the selected cells exposing the epitope sequence Peptide X leads to competitive interaction of AB with the Peptide X sequence in the cell surface protein

Fig. 5. Induction of $DiBAC_4(3)$ cellular uptake by different penetratin derivatives
A2 Medium
B3 Cells with $DiBAC_4(3)$
D7 10 µM penetratin-aminobezoic acid
B7 10 µM penetratin-biotin
F7 10 µM inactive penetratin

Fig. 6. Induction of $DiBAC_4(3)$ cellular uptake by different transportan derivatives
A2 Medium
B1 Cells with $DiBAC_4(3)$
D2 10 µM transportan-aminobezoic acid
F2 10 µM transportan-biotin
H1 10 µM inactive transportan

Fig. 7. Induction of $DiBAC_4(3)$ cellular uptake is dependent on CPP concentration
E2 Medium
F2 Cells with $DiBAC_4(3)$
H2 1 µM transportan-biotin
B5 5 µM transportan-biotin
D5 10 µM transportan-biotin
F5 20 µM transportan-biotin

Fig. 8. Impact of pVEC on the uptake of $DiBAC_4(3)$
A8 Medium
B8 Cells with $DiBAC_4(3)$
C8 5 µM pVEC
D8 10 µM pVEC
E8 20 µM pVEC
F8 30 µM pVEC
G8 40 µM pVEC

H8 50 µM pVEC

Fig. 9. Impact of Pro-TPb on the uptake of $DiBAC_4(3)$
A1 Medium
B1 Cells with $DiBAC_4(3)$
C1 5 µM Pro-TPb in medium
D1 5 µM Pro-TPb
E1 10 µM Pro-TPb
F1 20 µM Pro-TPb
G1 30 µM Pro-TPb
H1 40 µM Pro-TPb
A4 50 µM Pro-TPb in medium
B4 50 µM Pro-TPb

Fig. 10. Concentration-dependence of the fluorescence signal, $DiBAC_4(3)$
A2 0.2 µM $DiBAC_4(3)$ in medium
B2 0.2 µM $DiBAC_4(3)$
D2 0.4 µM $DiBAC_4(3)$
F2 0.6 µM $DiBAC_4(3)$
H2 0.8 µM $DiBAC_4(3)$
B5 1.0 µM $DiBAC_4(3)$

Fig. 11. Concentration-dependence of the fluorescence signal, peptide (TPb)
A8 Medium
C8 Cells with $DiBAC_4(3)$
E11 5 µM TPb
D11 10 µM TPb
C11 20 µM TPb
B11 30 µM TPb
A11 50 µM TPb

Fig. 12. Internalization of biotinylated YTA-2 in human colon adenocarcinoma, LoVo cells.A) Deteceted with streptavidin-FITC B) nuclear staining with Hoechst.

Fig. 13. Three basic functions: 1) selective cleavage (and thereby activation) by MMP-2 or MMP-MT1, 2) cellular penetration by peptides (CPPs) and 3) killing of nearby, preferably tumor cells or endothelia involved in tumor neovascularisation, by a known cytostatic agent.

Fig. 14. Internalisation of YTA-2 (A) compared to YTA-2ps (B) in LoVo cells, both biotynilated peptides detected by TRITC-avidin at 37°C.

Fig. 15. Method of determining the specific cleavage of YTA-2ps of the matrix metallo proteinase-2 (MMP-2). The substrate is being synthesised.

Fig. 16. Structure of 7TM spanning G-protein coupled receptor and trimeric G-protein.

Fig. 17. Cellular uptake of GOP (M569) in Bowes cells at 37°C

Fig. 18. Stimulation of insulin release in rat pancreatic islets by cell penetrating peptide GOP

Fig. 19A. Blood glucose concentrations in healthy rats after i.p. injection of GOP (100 nmol/kg) and glucose (1g/kg)

Fig. 19B. Plasma insulin levels in healthy rats after i.p. injection of GOP (100 nmol/kg) and glucose (1g/kg)

Fig. 20A. Internalisation of Prpc-avidin-FITC. 100 fold dilution of avidin-FITC together with 2.5 μM was incubated with Bowes melanoma cells for 3 h. The plasma and nuclear membrane is clearly outlined.

Fig. 20B. Translocation of 10μM peptide at 37 °C in LoVo cells (human colon cancer).

Fig. 20C. Translocation of 10μM peptide at 37 °C in LoVo cells (human colon cancer).

Fig. 21. Cellular internalisation in N2A cells at a concentration of 10μM A and B) Cells treated with APP 521-536 coupled to fluorescein. C) Cells treated with APP 725-740 coupled to fluorescein. D) Cells treated with PS-1 97-109 deletion analogue coupled to fluorescein. E) Cells treated with the known CPP penetratin coupled to fluorescein. F) Untreated cells.

Fig. 22. Localization of biotinyl-M511 (A), biotinyl-scrambled M511 (B), natural background (C) and penetratin (D) in Bowes melanoma cells. Concentration of the peptides was 10 μM, incubation time 1 h at 4°C. Staining was done using 150 nM streptavidin-FITC.

Fig. 23. Effect of M511 (upper and middle panel) and scrambled M511 (lower panel) on the contraction of the porcine left anterior descending coronary artery without epithelium (upper panel) and in the presence of intact epithelium (middle and lower panel). Contraction force is given in the arbitrary units. The meaning of the arrows: 1 - administration of 30 mM KCl; 2 – administration of substance P; 3 – washing-out; 4 – administration of 16 μM M511 or scrambled M511.

Fig. 24. Dependence of maximal observed relative contraction of porcine coronary artery on the concentration of M511 (■) and biotin coupled M511 (□). Contraction obtained by 90 mM KCl was taken as 100 %. Curves were obtained by nonlinear regression procedure

using Prism computer package (GraphPad Software Inc., USA), according to the dose-response equation with variable slope (Hill coefficient) but fixed bottom (0 %) and top (100 %) values.

Fig. 25. Effect of M511 (□- no preincubation of membranes with peptide; - • 25 min. preincubation of the membranes with peptide), biotinylated M511 (□ - no preincubation of membranes with peptide), and scrambled M511 (x - no preincubation of membranes with peptide) on the rate of GTPγS binding to the membranes prepared from the porcine left anterior descending coronary artery.

Fig.26. Effect of phospholipase C inhibitor U73122 on the contraction of the porcine left anterior descending coronary artery induced by M511. Contraction force is given in the arbitrary units. The meaning of the arrows: 1 - administration of 30 mM KCl; 2 – administration of substance P; 3 – washing-out; 4 – administration of 16 μM M511; 5 – administration of 30 μM U73122.

Fig 27A. GFP expression in N2A cells 3 days after transfection with unmodified 60 kDa PEI (A, B and C), or with TP10 modified (D, E and F). Concentration of plasmide was 0,5 μg/ well each time, N/P ratio is 4 (A and D), 8 (B and E) or 16 (C and F).

Fig. 27B. GFP activities measured on murine fibroblasts C3H 10T1/2. Concentration of plasmid in each case 1,2 μg/well. expression measured 24 h after transfection. B) luciferase activity shown relatively to control to background. Concentration of plasmid 0,5 μg/ well, protein expression measured 72 h after transfection.

Fig. 28. Positional scanning of CPP within human 7tm receptors.
Human 7tm receptor sequences was downloaded from the swissprot/trmbl databases. The sequences was searched for CPPs of the indicated length (8,12 and 17 aa long). The possition of the start of the CPP in the protein is divided by the total length of the protein, is plotted against the frequencey of occurense (fraction of CPPs). Here, a search window size of 17 aa produced the most hits. The four peaks in evidence correspondes the four intracellular parts of the 7tms, with the largest corresponding the IC3. It can be noted that the CPP functionality seems to correlate well, both with the topology of the 7tms, as well as the proposed G protein activation sites.

EXPERIMENTAL SECTION

Example 1

Prediction of cellular penetration properties of a peptide

Introduction

In most peptide quantitative structure activity relationship studies (QSAR), a set of dimensionless values is used to describe a composite of the physical characteristics of the amino acids (descriptors). In the classical literature 3 values, Z1, Z2 and Z3 are used for this purpose. Recently Wold and colleagues expanded this descriptor set with 2 more: Z4 and Z5; and produced descriptor scales covering 87 natural and non-natural amino acids.

Method

Using the expanded descriptor scales, bulk property values $\mathbf{Z}_\Sigma$ where assembled for 4 cell-penetrating peptides (CPPs): Transportan, penetratin, pVEC and MAP (the training set); and averaged over the total number of amino acids in the sequence. Here, the Z_3 value, mainly describing polarity, had the highest predicting power. A number of protein and random sequences where searched, for sequences falling within the bulk property value $\mathbf{Z}_\Sigma$ interval obtained from the training set.

The descriptor interval used where: $\mathbf{Z}_{\Sigma 1} < 0.2$ and $\mathbf{Z}_{\Sigma 2} < 1.1$ and $\mathbf{Z}_{\Sigma 3} < -0.49$ and $\mathbf{Z}_{\Sigma 4} < 0.33$ and $\mathbf{Z}_{\Sigma 5} < 0.95$ and $\mathbf{Z}_{\Sigma 5} > 0.12$.

Results

Searching either a random- or natural protein sequence, sequences corresponding to CPPs appear clustered in blocks. This behaviour is due to the existence of "transport motors", i.e. shorter sequences with CPP characteristics, in the search window. For the GLP-1 and Angiotensin receptors, sequences corresponding to CPPs were correctly predicted by the above outlined method. Searching a random sequence of 10,000 amino acid length returns on average 32 block hits for a sliding window length of 18 amino acids. However, the number of hits is dependent of window length. Other CPPs where used as controls. The criteria outlined above holds true for all of them, with the possible exception of the Tat/poly Arg family of CPPs.

Table 7 Example of block phenomena in the search of CPPs in AT1 receptor with an sliding window of 18 aa. The transport motor is the motif included in all sequences.

Position	Sequence	Block start
298	NPLFYGFLGKK**FKKYFLQ**	Transport motor
299	PLFYGFLGKKFKKYFLQL	
300	LFYGFLGKKFKKYFLQLL	
301	FYGFLGKKFKKYFLQLLK	

302	YGFLGKKFKKYFLQLLKY	
303	GFLGKKFKKYFLQLLKYI	
304	**FLGKKFKKYFLQLLKYIP**	M511
305	LGKKFKKYFLQLLKYIPP	
306	GKKFKKYFLQLLKYIPPK	
307	KKFKKYFLQLLKYIPPKA	
308	KFKKYFLQLLKYIPPKAK	
309	FKKYFLQLLKYIPPKAKS	
		Block end

Example 2

Peptide synthesis (describing defaultmethod of the experiments below if not indicated otherwise)

Peptides were synthesized in a stepwise manner in a 0.1 mmol scale on a peptide synthesizer (Applied Biosystems model 431A, USA) using t-Boc strategy of solid-phase peptide synthesis. tert-Butyloxycarbonyl amino acids (Bachem, Bubendorf, Switzerland) were coupled as hydroxybenzotriazole (HOBt) esters to a p-methylbenzylhydrylamine (MBHA) resin (Bachem, Bubendorf, Switzerland) to obtain C-terminally amidated peptide. Biotin was coupled manually to the N-terminus by adding a threefold excess of HOBt and o-benzotriazole-1-yl-N, N, N′, N′-tetramethyluronium tetrafluorborate (TBTU) activated biotin (Chemicon, Stockholm, Sweden) in DMF to the peptidyl-resin. The peptide was finally cleaved from the resin with liquid HF at 0°C for 30 min in the presence of p-cresol. The purity of the peptide was >98% as demonstrated by HPLC on an analytical Nucleosil 120-3 C-18 RP-HPLC column (0.4 × 10 cm) and the correct molecular mass was obtained by using a plasma desorption mass spectrometer (Bioion 20, Applied Biosystems, USA) (Langel, U., Land, T. & Bartfai, T. Design of chimeric peptide ligands to galanin receptors and substance P receptors. *Int J Pept Protein Res* **39**, 516-22. (1992)).

Cell culture (describing defaultmethod of the experiments below if not indicated otherwise)

Murine fibroblasts C3H 10T1/2 , mouse neuroblastoma N2A cells and COS-7 cells were grown in 10 cm petri dishes in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% fetal calf serum (FCS), 2mM L-Glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. The cells were seeded and replated every fifth day. COS-7 and 10T1/2 cells were trypsinized when seeded while the N2A cells were suspended by mechanical force by adding media to the cells. Before starting the experiments, the cells were grown to confluency and then seeded and diluted two times in media before adding to 24-well plates (approximatly 60 000 cells/well).

High throughput screening (HTS) of cargo delivery efficiency.

By using CPP-S-S-cargo constructs where the cargo is labelled with the 2-aminobenzoic acid fluorophore and the CPP with the 2-nitrotyrosine quencher (Fig. 5) it is possible to monitor, in real time, the intracellular degradation of the disulfide bond resulting from the

reducing intracellular milieu, and hence the cellular uptake of the constructs by increase in apparent fluorescence.

Screening the uptake of CPPs into cells

Insertion of CPP into the plasma membrane has in principle to modify the properties (fluidity, permeability etc.) of the membrane and transport through it. It has even been suggested that the cellular uptake of CPPs could be dependent on the membrane potential of cells and modify it. Therefore the present inventors firstly assessed whether the known cell-penetrating peptides influence the cellular uptake and equilibria of membrane potential sensitive dye $DiBAC_4(3)$.

$DiBAC_4(3)$ (bis-(1,3-dibutylbarbituric acid)trimethine oxonol) (Tang, W. *et al.* Development and evaluation of high throughput functional assay methods for HERG potassium channel. *J Biomol Screen* **6**, 325-31. (2001)) has been widely used for estimation of changes of the membrane potential in response to different stimuli. The fluorescence intensity of $DiBAC_4(3)$ increases three-fold upon the binding/internalisation to the cells. Depolarisation of cells increases the uptake of $DIBAC_4(3)$ and thereby also the fluorescence intensity, while the increase of the membrane potential leads to the decrease in the fluorescence intensity. Most of CPPs show tendency to accumulate in membranous structures of cells, therefore the fluorescence of $DiBAC_4(3)$ in the system monitors both the membrane potential as well as the changes of membrane properties.

Penetratin, transportan and their cell penetrating analogues led to marked increase in $DiBAC_4(3)$ cellular fluorescence but the respective inactive analogues had no impact on the fluorescence intensity allowing distinguishing between the penetrating and non penetrating peptides.

Materials and methods

Cells

K562 human erythroleukemia cells were cultivated in RPMI 1640 medium supplemented with 7.5% foetal calf serum and antibiotics by using standard cell culturing techniques.

DIBAC internalisation assay

The assay was carried through in black 96-well plates (Nunc) and the fluorescence intensity was recorded by using SPECTRAmax GEMINI XS dual scanning microplate spectrofluorimeter (Molecuar Devices Corporation, Sunnyvale, CA). Into each well of microplate, from 50,000 to 600,000 (optimal 100,000-300,000) K562 cells in serum-free RPMI were aliquoted, $DiBAC_4(3)$ was added from stock solution and the cells were incubated for 10-30 min (optimal 15 min) at 37 °C in CO_2 incubator to reach the equilibrial distribution of the dye between the cells and surrounding medium. After incubation, the respective peptides were quickly added to reach the concentration of 1-50 μM (usually 10 μM) for the peptide and 0.1-1 μM (in most cases 0.3-0.5 μM) and for $DiBAC_4(3)$. Each peptide or concentration was measured in triplicates in two independent experiments. The

intensity of the fluorescence was recorded every 30 sec during one hour by using SPECTRAmax GEMINI XS dual scanning microplate spectrofluorimeter using excitation at 480 nm and detecting emission at 520 nm. The cells in the wells were kept in suspension by shaking for 5 sec at the beginning of measurement and for 2 sec between every measurement. Data were analysed and the graphs were produced by using the programm package SOFTMaxPRO (Molecular Devices Corp.)

Results and discussion

Induction of DiBAC$_4$(3) cellular uptake by different transportan derivatives

Transportan induced strong increase of DiBAC$_4$(3) fluorescence intensity in the experimental setup, which was presumably caused by the increased uptake of DiBAC$_4$(3) by K562 cells (Fig.6). The redistribution of DiBAC$_4$(3) was rapid, recording of the fluorescence signal started about three minutes after the addition of peptides into suspension of K562 cells, but could not follow the initial increase of fluorescence intensity. The reporter group on transportan, biotin (F2) and aminobenzoic acid (D2) respectively, had also minute influence on the redistribution of DiBAC$_4$(3) between the solution phase and K562 cells, but this difference was not significant. The analogue of transportan that had a substitution of one proline for leucine in the C-terminal part of transportan (Pro-TP) (H1), which is known not to penetrate into cells, did not lead to any increase of DiBAC$_4$(3) fluorescence as compared to untreated cells (B1).

Induction of DiBAC$_4$(3) cellular uptake is dependent on CPP concentration

The induction of DiBAC$_4$(3) uptake was highly dependent on the concentration of cell-penetrating peptide. While low concentration of transportan induced only negligible and temporal increase in DiBAC$_4$(3) uptake (1 μM Fig.7, H2), high concentration of transportan induced remarkable pertaining increase in DiBAC$_4$(3) uptake (20 μM F5 Fig.7). The intermediate concentrations of transportan contributed accordingly into the increase of DiBAC$_4$(3) uptake (5 μM B5 and 10 μM D5 in Fig.7).

Induction of DiBAC$_4$(3) cellular uptake by different penetratin derivatives

Not only the cellular uptake of transportan, but also of penetratin could be followed by using DiBAC$_4$(3) (Fig.5). Again, active penetratin derivatives induced remarkable uptake of DiBAC$_4$(3) (B7 and D7 in Fig.5), while penetration deficient analogue (F7) did not. Some small increase in fluorescence intensity was observed at the beginning of the treatment of K562 cells with inactive penetratin, however, this could be caused by negligible uptake of this peptide.

pVEC peptide reduces the uptake of DiBAC$_4$(3)

In contrary to transportan and penetratin, the cell-penetrating peptide that was deduced from the sequence of murine VE-cadherin (pVEC), reduced the DiBAC$_4$(3) uptake. The drop in the fluorescence intensity that was induced by pVEC was smaller compared to the change induced by transportan or penetratin. However, the decrease of the fluorescence intensity was significant and strictly concentration dependent (Fig.8). The decrease of the

fluorescence intensity was not linearly dependent on the pVEC concentration, suggesting that saturation of the uptake may be encountered at higher concentration.

Non-penetrating analogue of transportan does not influence the uptake of DiBAC$_4$(3)

The analogues of CPPs with small substitutions, which did not enter the cells, probably did not strongly interact with the plasma membrane either. Indeed, the fluorescence intensity of DiBAC$_4$(3) was not significantly influenced by the presence of Pro-TP, an inactive analogue of transportan, in the cell suspension (Fig.9). The 5-20 μM concentration of Pro-TP lead to a negligible decrease of the fluorescence intensity (D1, E1, F1). Very high concentration (50 μM) of this peptide, however caused minute increase of fluorescence intensity. This increase could mostly be ascribed to the direct interaction of the dye with the peptide, since the analogous increase was observed also without the K562 cells, i.e. in RPMI 1640 medium (A4).

Sensitivity of the DiBAC$_4$(3) assay

The reliability of the DiBAC$_4$(3) based assay for distinguishing between the cell penetrating and non-penetrating peptides depends greatly on the magnitude of the signal i.e. the amplitude of the changes in the fluorescence intensity. In principle, the fluorescence intensity can be enhanced by increasing the concentration of DiBAC$_4$(3), peptide or the amount of cells. Therefore the inventors varied one of the respective parameters while keeping the two other constant. Increasing the number of cells per well from 50,000 to 400,000 at 10 μM peptide and 0.4 μM DiBAC$_4$(3) concentration respectively, yielded a gradual increase in fluorescence intensity. Further increase of the cell number didn't result in a remarkable gain of the fluorescence, pointing to the importance of relative amounts of cells, dye and peptide, rather than to absolute values in this aspect. In contrary, the fluorescence intensity was nearly linearly dependent on the concentration of DiBAC$_4$(3) in the studied range of 0.2-1 μM, when using 200,000 cells per well (Fig.10). The ratio between the intensities at 1 μM and 0.4 μM concentration of DiBAC$_4$(3), which was used in most experiments, is more than two-fold, showing that the sensitivity of the present assay can be remarkably enhanced by using higher concentration of DiBAC$_4$(3).

Most experiments were performed with a 10 μM concentration of transportan, but significantly higher fluorescence intensities were obtained at higher peptide concentration. Increasing the peptide concentration from 5 to 10 μM yielded in doubling the fluorescence signal (Fig.11). Further increase of transportan concentration lead to gradual increase of fluorescence up to 50 μM, which was the highest concentration to be assessed. However, such very high concentrations of transportan and DiBAC$_4$(3) can have adverse e.g. toxic and lytic effects on the cells and the obtained data have to be interpreted cautiously. The initial raise of fluorescence intensity for about 10 min, instead of the usually observed decrease at 40 and 50 μM, suggests that some cell lysis occurred at so high concentrations of transportan. On the other hand, the cell viability and the intactness of their plasma membrane could be easily assessed by supplementing the cell culture medium that was used in the assay with propidium iodide. Recording of propidium iodide fluorescence allowed simultaneous estimation of the proportion of cells with leaky plasma membrane.

Conclusion

In conclusion, this assay, which is based on the redistribution of the $DIBAC_4(3)$ between the cells in suspension and the cell medium allows distinguishing between the peptides which are and which are not taken up by the cells. Hence it can be applied for rapid screening of peptides for their ability to penetrate into cells.

Example 3

Sequence prediction

Several randomly generated sequences of 10.000 amino acids length were searched. The amount of hits varied with window length, but held at around 3% of the total sequences (see sequenses **151-x**). Around 500 G protein coupled receptor sequences were searched, about 0.015 % (see sequenses **151-x**)) of the possible sequences was found to mach the CPP criteria, perhaps demonstrating that CPPs are selected against in nature.
The criteria outlined, successfully predicted around 95% of published CPP sequences.

Example 4

Cell selective CPPs

Tissue specificity of CPPs:

As the cellular uptake of CPPs is likely to depend on membrane properties and membrane potential of the target cell, the possibility exists to obtain cell specific CPPs. Using the criteria outlined above, a biased library of peptides should be generated. The libraries will then be tested for uptake/cargo delivery efficiency using the method described by us in (Hallbrink et al). Briefly, by using CPP-S-S-cargo constructs where the cargo is labeled with the 2-aminobenzoic acid fluorophore and the CPP with the 3-nitrotyrosine quencher (Fig.12), it is possible to monitor (in real time) the intracellular degradation of the disulfide bond. Hence the cellular uptake of the constructs and the cargo delivery efficiency of the CPP can be measured by the increase in apparent fluorescence. This method eliminates the experimentally difficult step of distinguishing between internalised and outer membrane bound peptide.

Method of determining the specific cleavage of YTA-2ps of the matrix metallo proteinase-2 (MMP-2):

The substrate is being synthesised and the attachment and efficiency of the cytostatic agent to the CPP-part studied in parallel.
As illustrated in Fig.12, the inventors have been able to show that the CPP-part of a selective CPP (YTA-2) can efficiently enter cells both at 37° (Fig.12) and 4° C (data not shown). In addition, the "inactivator", see Fig.13, made the peptide less active in translocation over the cell membrane (Fig.14). The next step in the development of this technique is to check the specific cleavage of YTA-2ps by active MMP-2. It is performed by a fluorescence/quencher assay, where the MMP-2 substrate YTA-2ps upon cleavage will

increase in fluorescence intensity (Fig.15). The correct cleavage is also checked by mass spectrometry.

Example 5

Design of a functional protein mimicking CPP, examplified by a new effector-mimic-CPP for treatment of insulin deficiency in non-insulin dependent diabetes mellitus:

Background

GPCR-ligand interactions and their mimicry in disorders:

The interactions between 7TM spanning receptors with their respective G-proteins are well defined and specific. In fact, these interactions are the analogues of DNA/DNA interactions between specific and well-defined proteins. 7TM receptors are G-protein coupled receptors (GPCR) and as such, they expose amphipathic α-helical motifs, which are suggested to be responsible for G-protein binding. It has been demonstrated that parts of the GPCR's third intracellular loop, but sometimes also othe loops are involved in signaling. On the appended schematic drawing Fig.16, such a 7TM receptor-G-protein complex is presented. Synthetic peptides from the intracellular parts of GPCRs can mimic the interaction of the GPCR and G-protein, i.e. conveying an activated receptor signal, as has been demonstrated in cellular fragment systems.

In the present example, the inventors have demonstrated that a novel CPP, derived from one of said intracellular parts of a GPCR, can both act as a cell-penetrating peptide, as well as at the same time mimic the interaction of the GPCR and G-protein in the target cell.

Non-insulin dependent diabetes mellitus, NIDDM, and glucagon like peptide 1 receptor, GLP-1R

Non-insulin dependent diabetes mellitus, NIDDM, also known as type 2 diabetes mellitus, T2DM, is characterised by complex hormonal disturbances and insulin resistance. Treatment of NIDDM is complicated due to the complexity of the disorder, as well as to poor understanding of the mechanisms behind it. Probably, several key cellular and molecular mechanisms in NIDDM still remain to be defined. Despite the lack of comprehensive knowledge of mechanisms of NIDDM, recent achievements in diabetes research have revealed some promising targets for studies and treatment.

Non-insulin dependent diabetes mellitus, NIDDM, is currently treated by hormone replacement with insulin, with insulin releasers, or insulin sensitizers. However, none of these treatments is fully satisfactory in controlling serum glucose levels. Glucose dependent insulin release is partly controlled by the activation of the G-protein coupled receptor glucagon like peptide 1 receptor, GLP-1R, rendering it a promising target for a new NIDDM therapeutic agent. The existence of an ideal endogenous agonist for GLP-1R, the glucagon like peptide 1, GLP-1, has been known for almost 15 years. However, its pharmacological exploitation has so far failed due to short half-life of the peptide when administered i.v. and due to loss of agonist efficacy of most of the synthetic analogues.

The inventors herein show that GLP-1R agonist-mimics, based on intracellular loop 3 (IC3) peptides of G-protein coupled receptors can mimic the active state of the agonist occupied receptor in signalling to initiate insulin release.

The design and synthesis of peptides derived from GLP-1R IC3 are demonstrated, which induce insulin release in rat pancreatic islets. These approaches greatly facilitate the study of the mechanisms underlying the activation of insulin-release by GLP-1, and provide physical libraries of substances, which will stimulate insulin release, and thus serve as potent leads for the development of drugs for NIDDM treatment.

GLP-1R fragments as GLP-1 receptor agonist mimics.

Synthetic peptides derived from the third intracellular loop, IC3, of GLP-1 receptor express GTPase and adenylate cyclase (AC) activation with EC_{50} = 100 nM. The IC3 peptides are powerful enzyme activators, often a 6- to 13-fold activation of the AC is achieved. Additionally, these peptides may serve as tools for study of promiscuity of the GLP-1 receptor in signal transduction to G-proteins.

The short, 12-20 amino acids long IC3 peptides mimic the interactions of the agonist occupied 7TM receptor proteins *in vitro*. The peptides can be furthermore be connected to cellular transporters, such as transportan, for more efficient penetration into the cell interior where these interactions take place in *in vivo* studies.

Summary of results

The inventors have designed, synthesized and tested for insulin release a novel octapeptide derived from glucagon-like peptide-1 receptor, GLP-1R with sequence IVIAKLKA-amide (GOP). This peptide mimics the action of the parent peptide, GLP-1R, a seven transmembrane spanning protein known to initiate insulin release in pancreatic islets followed by recognition of the GLP-1 peptide hormone. Analogues of this peptide are novel cell-penetrating peptides, CPPs, that are able to translocate cell membranes in the tested cells. These data demonstrate a novel possibility to design agonists of a desired protein, which are cell penetrating by themselves.

Methods

Cellular penetration of biotin-labelled peptide

The medium containing serum was exchanged for a serum-free medium and water solution of the peptide was added directly into the medium to reach the concentration of 10 µM. A negative control was always used, because living cells all the time have some biotin inside. To control cells pure water instead peptide solution were added, and further handled alike all other. The cells were incubated for 1 h at 37°C or 4°C in 5% CO_2 enriched air. The cells were washed three times with PBS, fixed and permeabilised with methanol for 10 min at –20°C, washed again with PBS and incubated for 1 h in a 5% (w/v) solution of fat-free milk in PBS in order to decrease non-specific binding. The peptides were visualised by staining with 0.1 µM streptavidin-FITC in the same solution for 1 hour at room temperature. The

cell nuclei were visualised by staining with DNA with Hoechst 33258 (0.5 µg/ml) for 5 min, thereafter the coverslips were washed 3 times with PBS and mounted in 20 % glycerol in PBS. The images were obtained by Zeiss Axioplan 2 microscope (Carl Zeiss Inc., Germany).

Insulin release

Effects of the peptides on insulin secretion were assessed in pancreatic islets from male Wistar rats weighing 200-250 g. Islets were isolated aseptically by collagenase digestion, and then cultured overnight at 37°C in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum. After culturing, analysis of insulin secretion was performed by incubation at 37°C for 1 h in batches of 3 islets, each in 300 µl of Krebs-Ringer bicarbonate buffer with 10 mM Hepes and 2 mg/ml bovine serum albumin, pH 7.4. The incubation medium contained either 3.3 or 16.7 mM glucose, with or without peptide. In one experimental series, batches of $5x10^5$ Rin m5F cells were incubated under similar conditions, except that glucose was omitted from the medium. Aliquots of the incubation medium were taken for radioimmunoassay of insulin. Insulin secretion is expressed as µU insulin/islet/h and µU insulin/$5x10^5$ cells/h, respectively. Statistical significance was evaluated with the Student's *t*-test with $p<0.05$ regarded as significant

Results and conclusions

A short peptide derived from the GLP-1R-sequences is able to dose-dependently and glucose dependendently increase insulin secretion form isolated rat pancreatic islets. Furthermore the peptide is able to increase insulin secretion and decrease blood-glucose levels when injected i.v. in rats.

Cell penetration of GLP-1R loop derived peptides

In Fig.17, the cellular penetration of the GOP analogue M569 is illustrated. The inventors have been able to further develop the GLP-1R derived peptides described above. A new generation of these peptides, which includes the sequence IVIAKLKA is characterized to have cell penetrating properties, activate G-proteins and increase insulin release when incubated with rat pancreatic islets. The GLP-1R derived peptide M569 shows temperature independent penetration into the human Bowes melanoma cells and the same intracellular localization of the peptide is registered at 4°C as well (not shown).

Insulin release

In the absence of the peptide GOP, 16.7 mM glucose stimulated insulin release almost 3-fold as compared to basal release at 3.3 mM glucose (Fig.19). GOP (10 µM) stimulated insulin release 5-fold ($p<0.001$) at 3.3 mM glucose, and at 16.7 mM glucose, the stimulation by 1 and 10 µM GOP is 4-fold.

In vivo effect of GOP (M528) on insulin release and blood glucose level

Healthy male Wistar rats, weighing approximately 250 g, were fasted over-night. After an initial blood sample obtained by incision of the distal tail vein (0 min), GOP (M528, 100 nmol/kg; n=4) was injected intraperitoneally, followed by another i.p. injection of glucose (1 g/kg). Control rats (n=4) were injected with saline and glucose. Additional blood samples were taken from the tail vein after 10 and 30 min. Blood glucose levels were determined by a glucose oxidase method and plasma insulin levels by radioimmunoassay.

As evident from Fig.19A and Fig.19B, plasma insulin and blood glucose concentrations were similar in all rats before injections. After 10 min, plasma insulin had increased significantly in the GOP-injected rats, compared to control rats ($p<0.05$) and to same group of rats at 0 min ($p<0.01$) (Fig.19A). In parallel, blood glucose levels were significantly lower in the GOP-injected rats relative to control rats at 10 min ($p<0.02$) (Fig.19B). After 30 min there were no differences between the two groups of rats regarding insulin and glucose levels. The present data indicate a marked, however transient, effect of the peptide on insulin release *in vivo*. In conclusion, the GLP-1R derived peptides of the GOP family penetrate cell membranes, interact specifically with respective G-protein and increase insulin release from rat pancreatic islets.

Table 8: GOP-peptide derivatives

Peptide	**Sequence**
GOP	IVIAKLKA amide
GOP2	$(CIVIAKLKA)_2$ amide
GOP3	IVIAKLRA amide
GOP4	IAIAKLKA amide
GOP5	IVIAKLAA amide
GOP6	all-D-(IVIAKLKA) amide
GOP7	I-(N-Me-V)-IAKLKA amide
GOP8	I(all-N-Me(VIAKLKA)) amide
GOP9	IV-Oca-KA amide
GOP10	AKLKAIVI amide
GOP11	IAIAKLAA amide
GOP12	VIAKLK amide
GOP13	IVI-(N-Me-A)KLKA amide
GOP14	IVIAKLK-(N-Me-A) amide
GOP15	IVI-Aib-KLKA amide
GOP16	IVVSKLKA amide
GOP17	IVIAKLKA-COOH
GOP18	InorVIAKnorLCitA-COOH

Series of GOP analogues will be chosen among the following:

IVV-X-KLKA, IVI-X-KLKA, IV-X_1-X-KLKA

Wher X_1 is an amino acid and X is a linker.

Example 6

Design of a non-receptor functional protein mimicking CPP, examplified by a new effector-mimic-CPP corresponding to the N-terminal sequences of the prion protein (PrpC)

Background

The N-terminal sequences of the prion protein (PrPC) are very similar to constructed signal peptide-NLS chimera, shown to function as cell-penetrating peptides (CPPs) (Vidal, P. *et al.* Interactions of primary amphipathic vector peptides with membranes. Conformational consequences and influence on cellular localization. *J Membr Biol* **162**, 259-64. (1998)). Based on these sequence similarities, the inventors tested the hypothesis that also the PrPC derived sequence from mouse with non-cleaved signal sequence is active as a CPP. The inventors found that mouse PrPC(1-28) is indeed a CPP, with the ability to carry a cargo avidin into cells, based on a standard fluorescence assay technique. In distilled water, the PrPC(1-28) peptide is strongly aggregated above 1 mM concentration, and has a dominating β structure. The findings have significant implications for the understanding of how prion proteins with intact N termini may invade cells and of the secondary structure conversion to β structure that is associated with the conversion to the scrapie form of the protein.

The following peptide sequences were compared:
The chimeric CPP, i.e. the hydrophobic sequence from gp41 of HIV (1-17) + NLS from SV40 large antigen T (18-24): MGLGL HLLVL AAALQ GAKKK RKVC (1)
Mouse PrpC(1-28): MANLG YWLLA LFVTM WTDVG LCKKR PKP (2)
Human PrpC(1-28): MANLG CWMLV LFVAT WSDLG LCKKR PKP (3)
Bovine PrPC (1-30): MVKSK IGSWI LVLFV AMWSD VGLCK KRPKP (4).

The general features of both types of sequences is a mainly hydrophobic signal peptide part of 16-23 residues followed by a NLS part of about 7 residues, with high positive charge.

Mouse PrpC(1-28) was synthezised with a biotin lable in the N terminus to investigate its cell penetrating properties. The internalisation of the peptide was monitored through the coupled flourescine, as previously described in example 7 and conducted in cultured N2A cells. The cell penetration properties were investigated for the peptide itself and for the peptide carrying a large cargo of the avidin protein (65 kD). The protocol followed was the same as used in previous experiments (Kilk, K. *et al.* Cellular internalization of a cargo complex with a novel peptide derived from the third helix of the islet-1 homeodomain. Comparison with the penetratin peptide. *Bioconjug Chem* **12**, 911-6. (2001), Magzoub, M., Kilk, K., Eriksson, L.E., Langel, U. & Graslund, A. Interaction and structure induction of cell-

penetrating peptides in the presence of phospholipid vesicles. *Biochim Biophys Acta* **1512**, 77-89. (2001)) demonstrating CPP properties of the pIsl peptide sequence derived from the homeodomain of the rat transcription factor Islet-1 (Inoue, A., Takahashi, M., Hatta, K., Hotta, Y. & Okamoto, H. Developmental regulation of islet-1 mRNA expression during neuronal differentiation in embryonic zebrafish. *Dev Dyn* **199**, 1-11. (1994)). Figure 20A shows fluorescence microscope pictures clearly indicating the CPP efficiency and perinuclear localization of both preparations, PrpC(1-28) without and with the avidin cargo.

The secondary structures of the peptide were further investigated, now without biotin attachment, in aqueous solution and in various membrane mimetic solvents by CD and NMR spectroscopy. Figure 20B shows a CD spectrum of 1 mM peptide in distilled water, with features typical of a significant β structure contribution. Addition of salt increased the β structure contribution (data not shown). Parallel ^{1}H NMR experiments yielded no evidence of a resolved spectrum and attempts to investigate diffusion showed that peptide aggregates had formed in the sample that were larger than could be measured by the NMR technique. In the presence of negatively charged phospholipid vesicles the β structure contribution of the peptide was considerably increased (Figure 20B). Figure 20C shows a partial NMR TOCSY spectrum of the peptide in SDS micelles, which dissolves the peptide aggregates so that a well resolved 1H NMR could be obtained and the resonances assigned. The secondary chemical shifts of the Hαs along the peptide chain give clear evidence of induced α-helical structure. This chameleon-like behaviour of the peptide in various solvents essentially mirrors observations from other CPPs, like penetratin (Derossi, D., Joliot, A.H., Chassaing, G. & Prochiantz, A. The third helix of the Antennapedia homeodomain translocates through biological membranes. *J. Biol. Chem.* **269**, 10444-10450. (1994)) and pIsl (Kilk, K. *et al.* Cellular internalization of a cargo complex with a novel peptide derived from the third helix of the islet-1 homeodomain. Comparison with the penetratin peptide. *Bioconjug Chem* **12**, 911-6. (2001)) except that the aggregation and β structure contribution in aqueous solution is more pronounced and even more dependent on ionic strength and peptide concentration for mouse PrPC(1-28) than for penetratin and pIsl.

Example 7

Design of a functional protein mimicking CPP, examplified by new effector-mimic-CPPs corresponding to amyloid precursor protein (APP) and presenilin-1 (PS-1)

Background

Alzheimer's disease is the most common form of dementia in the elderly, and although the disease was discovered almost a century ago, no cure nor exact mechanism of action have been discovered. The most classic hallmark of the disease is protein aggregates, called senile plaques. These senile plaques consist mainly of a peptide derived from the amyloid precursor protein, called β-amyloid. This β-amyloid peptide is created during the processing of the amyloid precursor protein, where two proteins called presenilin-1 and presenilin-2 are thought to be involved.

Summary

The example demonstrates that the amyloid precursor protein (APP) and presenilin-1 (PS-1) have specific peptide sequences, so called cell-penetrating sequences, which can help them to translocate across cell membranes and be internalised by living cells. This might be used as a mechanism by which secretory APP, or any other neuroprotective peptide, can be internalised and thereby exert its neuroprotective properties.

Materials and methods

Cellular assays

Cell culture:

Mouse neuroblastoma cells, N2A, were cultivated in Dulbecco's minimal essential medium with Glutamax-I, supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml).

Cellular internalisation assay:

The cells used for internalisation were seeded out on round glass coverslips in 24-well plates. One day post seeding, the cells were semi confluent, and the medium was changed to serum-free medium. The fluorescein-labelled peptides were added, with a final concentration of 10 µM. After 60 min of incubation at 37 °, the cells were washed 3 times with 1 ml of Hepes-Krebbs-Ringer-(HKR) buffer and fixed with 3% paraformaldehyd solution in phosphate-buffered saline solution (PBS) for 10 minutes. The cells were then washed 3 times with 1 ml of HKR-buffer, and the coverslips were mounted and sealed for microscopy studies.

Results

Peptide synthesis and purification

The following peptides were synthesised, coupled with fluorescein, purified on HPLC and analysed on mass spectrometer (as described in Langel, U., Land, T. & Bartfai, T. Design of chimeric peptide ligands to galanin receptors and substance P receptors. *Int J Pept Protein Res* **39**, 516-22. (1992)).

Table 9. Sequence, calculated molecular masses and measured molecular masses of synthesised APP and PS peptides labeled with fluorescein

Peptide	Sequence
APP (521-537)	KKAAQIRSQVMTHLRVI
APP (712-726) WT	IATVIVITLVMLKKK
APP (712-726) mutant V717F	IATVIFITLVMLKKK
APP (725-740)	KKKQYTSIHHGVVEVD
PS-2 (86-110)	KVHIMLFVPVTLCMIVVVATIKSVR

PS-1 (151-162)	VVLYKYRCYKVI

Cellular internalisation assay

The cell type used for the internalisation assays with the fluorescein tagged peptides was N2A mouse neuroblastoma cells. This cell-line is commonly used in association with Alzheimer's disease studies, and serves as a good model cell-line for these internalisation assays where peptides derived from proteins involved in Alzheimer's disease were examined. These internalisation assays were performed at 37°C, therefore the internalisation by endocytosis cannot be excluded.

Fig.21 shows cellular localisation of peptides in N2A cells at a concentration of 10μM A) and B) Cells treated with APP 521-536 labeled with fluorescein. C) Cells treated with APP 725-740 labeled with fluorescein. D) Cells treated with PS-1 97-109 deletion analogue coupled to fluorescein. E) Cells treated with the known CPP penetratin labeled with fluorescein. F) Untreated cells.

The internalisation experiments demonstrate that APP (521-536), which is derived from the extracellular part of the protein, has cell-penetrating abilities (Fig.21 A and B), localising both in the nucleus and the membrane. This is a putative pathway by which the secretory amyloid precursor protein (sAPP) is internalised. This fact is interesting since this fragment has been shown to protect neurons against hypoglycemic damage and glutamate neurotoxicity thus acting as neuroprotective agent. The presenilin-1 (97-109) deletion analogue, derived from a membrane spanning- and first extracellular loop part, also showed cell-penetrating abilities. However, this peptide is mostly localised in the cytosole, but can also be detected in the plasma-membrane and nucleus (Fig.21D). APP (725-740) shows an increased fluorescence compared to the background, which seems to be situated mostly to the cytosol. However, this does not seem as clear as in the earlier mentioned peptides, and recent experiments have shown that it is not internalised at 4°C , which suggests that the slight increase in fluorescence that was observed is probably caused by endocytosis. Identical increase in fluorescence could also be observed for presenilin-1 (151-162) (not shown), which is a peptide derived from the first intracellular loop in presenilin-1. The internalisation experiments with APP (712-726) WT and APP (712-726) V717F mutant, do not reveal any no uptake. The reason behind this is probably that as soon as these peptides were added to the 24-well plate, they formed aggregates. This tendency to aggregate was also observed earlier, especially when the crude product was dissolved for purification on HPLC.

Example 8

Design of a functional protein mimicking CPP, examplified by a new effector-mimic-CPP corresponding to a synthetic peptide derived from the intracellular C-terminus of angiotensin 1A receptor.

Example 8 discloses a novel vasoconstrictor, more precisely to a synthetic peptide derived from the intracellular C-terminus of angiotensin 1A receptor. The peptide is a cell-penetrating peptide and promotes contraction of heart coronary blood vessels.

Background

Angiotensin receptors are members of the 7-transmembrane G-protein coupled receptor family and are important components of the blood pressure and electrolyte balance maintaining system in mammals. They exist in two types: AT1 (consists of AT1A and AT1B subtypes) and AT2, among which AT1 seems to be responsible for the mediation of almost all known systemic effects of angiotensin II. AT1 receptors are involved in contraction of smooth muscles in different tissue, e.g. in blood vessels, uterus, bladder, and some endocrine glands, and are widely distributed in kidney, liver, and in CNS. Antagonists of AT1 receptor are potential antihypertensive drugs and some non-peptide antagonists, e.g. lorsatan, have been successfully introduced in clinical use. Selective agonists for AT1 receptor, however, are not available today. Agonists would be of interest as potential drugs useful in the situations where vasoconstriction is required, e.g. chronical hypotension or migraine.

Methods and Materials

Cell culture

Bowes melanoma cells (American Type Culture Collection CRL-9607) were cultivated in Minimal Essential Medium (MEM, Life technologies, Stockholm, Sweden) with glutamax supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin solution, 1% non-essential amino acids and 1% sodium pyruvate.

Cellular internalisation assay

The cells used for internalisation experiments were seeded at a density of 10.000 cells/well on round glass coverslips in 24-well plates. After one day, when they had reached about 50% confluency, the medium was changed to serum-free and biotinylated peptides were added directly into the medium. After 60 min of incubation at either 37°C or 4°C, the cells were washed three times with PBS and fixed with 3% (w/v) paraformaldehyde solution in PBS for 15 min. For indirect detection of the biotin labeled peptide, the fixed cells were permeabilized with 0.5% Triton X-100 in PBS and sites for non-specific binding were blocked with 5% BSA in PBS. The biotin moieties were visualized by incubation of the treated cells with streptavidin-TRITC (Molecular Probes, Netherlands, 1:200) for 1 h at room temperature. The cell nuclei were stained with Hoechst 33258 (0.5 µg/ml, Molecular Probes, Holland). The fluorescence was examined by using a Zeiss Axioplan 2 microscope (Carl Zeiss AB, Sweden) equipped with a CCD (C4880, Hamamatsu Photonics, Japan).

Tissue preparation

Dissected porcine hearts (260 – 390 g) were transported from local slaughterhouse to the laboratory in ice-cold Krebs-Henseleit solution (119 mM NaCl, 23.8 mM $NaHCO_3$, 3 mM KCl, 1.14 mM NaH_2PO_4, 1.63 mM $CaCl_2$, and 16.5 mM glucose). Left anterior descending coronary artery and great cardiac vein were isolated. Part of each blood vessel was used immediately after isolation for contraction assays while part of it was kept frozen in liquid nitrogen for the membrane preparation. In some experiments blood vessels in which endothelium was mechanically removed were used. The same procedure was used also for the preparation of human umbilical blood vessels; umbilical cords were obtained from Obstetric Clinic of Ljubljana Clinical Center, Slovenia.

Blood vessel contraction measurement

The measurements were performed as already described **(20)**. Rings (5 mm wide) of blood vessels were cut and mounted into 10 ml tissue chamber filled with Krebs-Henseleit solution (37°C; pH=7.4) which was oxygenated with the mixture of 95% O_2 and 5% CO_2. Initial tension of 50 mN was applied and after equilibration 30 mM KCl was added to obtain stable isometric contraction. The presence of endothelium was verified with substance P. After washing out of KCl and substance P and after the equilibration of the system was restored, test peptide was added into the tissue chamber and blood vessel contraction was recorded.

Overexpression of G-proteins in Sf9 cells

Baculovirus transfer vectors harboring the genes for Gsα, Giα1 and β1γ2 were kindly provided by Dr. Tatsuya Haga (University of Tokyo, Tokyo, Japan). The cDNA for bovine Gsα (21) in pVL1392, bovine Giα1 (22) in pVL1392 and bovine β1γ2 (23) in pVL1393 were cotransfected with linearized baculovirus DNA (Pharmingen, San Diego, CA, USA), and the resulted virus stocks were subjected to one round of plaque purification before generation of high-titer virus stocks. In the expression procedure, Sf9 cells at density of 2 million cells/ml in suspension culture were infected with a ratio 2:1 of α versus βγ. Cells were harvested 48h post-infection, washed in PBS and stored at –70 °C until membrane preparation.

Membrane preparation

Frozen pieces of blood vessels were mechanically pulverized. Subsequently membranes were obtained according to the protocol of McKenzie (McKenzie, F.R. *Signal Transduction* (Milligan, E., Ed.), Oxford University Press, Oxford, NY, Tokyo. (1992)) with minor modifications. Until used, they were kept frozen in the concentration of 1 - 2 mg protein/ml as determined by the method of Lowry (Lowry, O.H., Rosenbrough, n.Y., Farr, A.L. & Randall, R.J. *J. Biol. Chem.* **193**, 265-275 (1951)). The same procedure (with the exception of pulverization of tissue) was used also for the preparation of membranes from Sf9 cells which overexpressed the G-proteins of different types; protein concentrations of these membrane preparations were between 0.2 and 0.4 mg/ml.

The rate of GTPγS binding

The binding to G-proteins from blood vessel membranes was followed as described by McKenzie(McKenzie, F.R. *Signal Transduction* (Milligan, E., Ed.), Oxford University Press, Oxford, NY, Tokyo. (1992)). Briefly, the membranes (final protein concentration in the assay mixture was 0.05 mg/ml) were incubated for 3 min in the absence and presence of peptides in different concentrations with 0.5 nM [^{35}S]GTPγS at 13°C in TE-buffer (10 mM Tris-HCl, 0.1 mM EDTA), pH 7.5. The unbound [^{35}S]GTPγS was removed by rapid filtration of the reaction mixture through Millipore GF/C glass-fiber filters under vacuum. The remaining radioactivity contained in the filters was determined in the LKB 1214 Rackbeta liquid scintillation counter. Blank values were determined by replacing the membranepreparations with buffer.

Measurement of GTPase activity

The measurements were performed radiometrically according to Cassel and Selinger (Cassel, D. & Selinger, Z. (1976) **452**(2), 538-51. **452**, 538-551 (1976, with the modifications suggested by McKenzie (McKenzie, F.R. *Signal Transduction* (Milligan, E., Ed.), Oxford University Press, Oxford, NY, Tokyo. (1992)). To the diluted membranes obtained from Sf9 cells with the overexpressed G-proteins of different types (final protein concentration in the assay mixture was 0.01 mg/ml) the ice cold reaction cocktail containing ATP (1 mM), 5'-adenylylimido-diphosphate (1 mM), ouabain (1 mM), phosphocreatine (10 mM), creatine phospho-kinase (2.5 Units/ ml), dithiothreitol (4 mM), $MgCl_2$ (5 mM), NaCl (100 mM), and trace amounts of [γ-^{32}P]GTP to give 50.000 - 100.000 cpm in an aliquot of the reaction cocktail (with the addition of cold GTP to give the required 0.5 µM total concentration of GTP) was added. Incubation medium was standard TE-buffer, pH 7.5. Background low-affinity hydrolysis of [γ-^{32}P]GTP was assessed by incubating parallel tubes in the presence of 100 µM GTP. Blank values were determined by the replacement of membrane solution with assay buffer. GTPase reaction was started by transferal of the reaction mixtures to 30°C water bath for 20 min. Unreacted GTP was removed by the 5% suspension of the activated charcoal in 20 mM H_3PO_4. The radioactivity of the yielding radioactive phosphate was determined in Packard 3255 liquid scintillation counter.

Curve fittings and other calculations as well as graphical presentations of the results were done by using a Prism computer program (GraphPad Software Inc., USA).

Results

Cellular internalisation of peptides

Biotinylated M511 internalized into living cells at both 4°C and 37°C, as judged by indirect immunofluorescence. The peptide translocates in a temperature-independent manner into Bowes melanoma cells and therefore, the main mechanism of uptake could not be endocytosis. The peptide localized preferentially in nuclei (Fig.22A) but also in the cytoplasm. Scrambled analogue of M511 (also biotinylated) was found to internalize into Bowes cell yielding a similar cellular localization and temperature dependence with M511

(Fig.22B), with, however, slightly lower efficiency. Penetratin a well-studied cell-penetrating peptide (12-14) was used as positive control (Fig.22D).

Blood vessel contraction

It was observed that M511 and biotinylated M511 act as powerful contractors of blood vessels. As seen in upper panel of Fig.23, M511 at 16 µM concentration promotes intense and long-lasting contraction of porcine left anterior descending coronary artery. The strength of contraction is comparable to the effect of 30 mM K^+ that is approximately 50 % of maximal effect of potassium ion. With higher concentrations the maximal effect approaches the effect of 90 mM KCl that is generally considered to be a maximal attainable contraction effect. Contrary to the contractory effect of potassium, the effect of M511 could not be terminated by washing the contractor out of assay solution. Furthermore it showed a concentration dependent 5 to 15 min lag-period after addition of M511 before contraction occurred. Results (Fig.23 middle panel) also show that blood vessel endothelium is not essential for the effect of M511.

Qualitatively the same but quantitatively more pronounced blood vessel contraction as shown in Fig.23 for M511 was observed also with biotin coupled M511 (diagram not shown). Concentration dependency of the maximal contraction force of porcine left anterior descending coronary artery exerted by M511 and biotin labeled M511 is shown in Fig.24 Scrambled M511 under same circumstances as M511, did not cause any contraction (Fig. 23 bottom panel).

GTPγS binding

As presented in Fig.24, the rate of binding of GTPγS to the membranes obtained from the porcine left anterior descending coronary artery was dose-dependently increased in the presence of M511, whichwas even more pronounced in the presence of biotinylated M511. The upper plateau of the effect was not obtained since the experimental points in the presence of peptides in concentrations over 500 µM could not be used. It is obvious that this effect is sequence specific for M511 (and also biotinylated M511) since the scrambled peptide is not active. Principally the same results were obtained also with membranes prepared from porcine great coronary vein and human umbilical artery (diagrams not shown).

G-protein selectivity

In order to shed some light on the type of G-proteins that were affected by M511, the inventors used membranes from Sf9 cells overexpressing G-proteins of different types and measured GTPase activity of these membranes in the absence and presence of M511 (100 µM). The results summarized in Fig.24 show small but significant activation of G_i and G_o and, interestingly, also moderate inhibition of G_{11}; G_s type of G-proteins seems not to be affected. These findings are in accordance with the suggestion that AT1A receptors function via inhibition of adenylyl cyclase (activation of G_i/G_o) and via modulation of phosphoinositide metabolism, most probably through pertussis toxin insensitive G-proteins

(G_q and G_{11}). Indeed was demonstrated regulation of G_{11} by M511, however, not by activation of this type of G-proteins but rather by their inhibition.

Conclusions

M511 is a peptide corresponding to rat AT1A receptor positions 304-327 (Table 7). As Fig. 22 demonstrates, biotinylated M511 penetrates into human melanoma cell-line Bowes similarly to a well-studied cell-permeable peptide Penetratin. Moreover, results obtained by porcine artery and vein vessel contraction, confirm internalization of M511 and suggest, that the internalized peptide may compete with native receptor and affect its signaling pathway. Interestingly scrambled biotinylated M511 internalize as well into Bowes cells, but does not cause contraction in artery or vein vessel. The observed lag-period in muscle contraction studies could be interpreted as time required for the penetration of sufficient amount of the peptide into the cell and the shortening of lag-period with the increasing concentration of peptides, as well as the inability to terminate the contraction by washing M511 from the assay solution, would be in accordance with its intracellular action. Virtually identical effect of M511 in porcine artery and great cardiac vein and human umbilical artery, illustrate that this effect is not restricted only to arteries and indicating its general nature in blood vessels of different tissues.

It is well known that AT1A as a member of 7-transmembrane receptors, is coupled to G-proteins via interaction of G_α subunit and the intracellular parts of the receptor. Increased rate of GTPγS binding (Fig.25) proves the involvement of G-proteins in the process of blood vessel contraction induced by M511 and corroborate the idea that M511 uncouples G-proteins from the AT_{1A} receptors. The presented results match well with the effect of the peptides on blood vessel contraction (see Fig.23 and 24).

In order to elucidate which G-proteins might be involved in the action of M511, GTPase activity in membranes overexpressing different types of G-proteins was measured. Slight activation of G_i/G_o and no effect on G_s is in good accordance with previous studies.

The inventors further inspected the mechanism of M511 action by using phospholipase C inhibitor U73122. As seen in Fig.26, U73122 at 30 μM concentration did not affect tonus of the porcine left anterior descending coronary artery (middle panel), and also did not modify blood vessel contraction induced by 16 μM concentration of M511 after posterior administration (upper panel), but it substantially decreased (for more than 50%) the effect of M511 when added 30 minutes prior to M511 administration (lower panel). This indicates that blood vessel contraction by M511 is mediated via phosphoinositole phosphate mechanism, as expected.

Another intriguing discovery is amplification of the effect of the peptide on blood vessel contraction via biotinylation. It can be proven by principally same effect on GTPγS binding rate (Fig.25). GTPγS binding rate demonstrates also that N-terminal biotin on M511 does not induce a parallel signaling cascade, leading to contraction, but rather amplifies interactions between G-proteins and the peptide.

In conclusion, M511 (and even more remarkably, biotinylated M511) seems to be a powerful vasoconstrictor that successfully penetrates the cells and functions via uncoupling of AT1A receptors from G_i and G_o proteins, and possibly also via inhibiting G_q proteins. As such it is an interesting drug candidate aimed against chronical hypotension and possibly also migraine. Its potential disadvantage is relatively high concentration required for the blood vessel contraction but its advantage could be its spontaneous internalization into the cells and its long-lasting action. Besides that it gives a new quality for angiotensin studies.

Summary of results

The inventors studied angiotensin and signal transduction via its receptors. By investigation of cell-penetrating peptides, they succeeded to design a peptide that penetrates cell membranes and induces *in vivo* intracellular signaling cascade similarly to the AT1A type of angiotensin receptor. The peptide, M511, corresponding to the fragment 304-327 of rat AT1A receptor, was found to internalize into Bowes cells in a temperature independent manner. This observation was confirmed by contractions of blood vessels from different origins. Induction of long lasting contraction of porcine coronary artery vessel and vessels from several other origins, after concentration dependent lag-period was assigned for the solution of the peptide. In order to discover principles of this action, influence on GTPγS binding rate and Gα-subtype selectivity of the peptide were measured. Results indicate, that the M511 peptide interacts with same selectivity to G-protein subtypes as agonist activated AT1A receptor, activating/inhibiting them. Down-regulation of blood vessel contraction by U73122 indicates that the further pathway involves phosphoinositole phosphate system and stimulates phospholipase C for M511 was observed also with biotin coupled M511 (diagram not shown). Concentration dependency of the maximal contraction force of porcine left anterior descending coronary artery exerted by M511 and biotin labeled M511 is shown in Fig.24. Scrambled sequence M511 under same circumstances as M511, did not cause any contraction (Fig.23 bottom panel).

Example 9

Combining the effects of PEI and TP10

In this study the inventors developed a new gene delivery system based on already existing PEI protocols. By combining the effects of PEI and TP10 together substantially higher transfection ratios were achieved than with PEI only. Two different approaches were presumed in order to create this system: TP10 with a specific PNA-anchor was hybridized to reporter gene plasmids and TP10 was crosslinked to transfection reagent. Thereafter plasmid or PEI of common transfection protocol was replaced by TP10 modified one, and no more changes in protocol were done. Under optimal conditions, the results from both experimental settings postulate a significant improvement in gene delivery compared to other systems.

Materials and Methods

Synthesis and purification of TP-10

TP10 was synthesized in a stepwise manner on an Perkin Elmer/ Applied Biosystem Model 431A peptide synthesizer, using t-Boc strategy according to protocol described previously (Langel, Land et al. 1992). Cysteine or glutamic acid was coupled manually. TP10 sequence is given in Pooga, 1998, FASEB J.. Prior conjugating peptides to PEI, TP10 was purified on a reversed phase HPLC (Gynkotek) C18 column with AcN/H_2O gradient, and analyzed using a MALDI-TOF Mass spectrometer (Applied Biosystems model Voyager STR). The mass values obtained matched calculated values.

PEI modifications

Conjugation of TP10 to PEI was done in two alternative ways. In the first case, a cysteine was coupled to Lys7 side chain. PEI (1mg/ml, MW: 60 kDa, Aldrich) was treated with bifunctional crosslinker succinimidyl trans-4-(maleimidylmethyl)cyclohexane- 1-carboxylate (SMCC) at concentratios needed for different TP10/PEI molar ratios. In the second case, glutamic acid was coupled to the N-terminus of TP 10 and was further covalently coupled to PEI (MW: 25 kDa, Sigma) using BOP generated Hobt esters. The calculated molecular weight of formed complexes was confirmed by MALDI TOF mass-spectrometry.

Cell culture

Murine fibroblasts C3H 10T1/2 , mouse neuroblastoma N2A cells and COS-7 cells were grown in 10 cm petri dishes in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% fetal calf serum (FCS), 2mM L-Glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. The cells were seeded and replated every fifth day. COS-7 and 10T1/2 cells were trypsinized when seeded while the N2A cells were suspended by mechanical force by adding media to the cells. Before starting the experiments, the cells were grown to confluency and then seeded and diluted two times in media before adding to 24-well plates (approximatly 60 000 cells/well).

Propagation of plasmids

pEGFP-N1, pEGFP-C2 (both Clontech), pGL3-Promoter Vector (Promega) and pRL-CMV Vector (Promega) plasmids were propagated in replication competent E.coli, according the protocol suggested by the manufacturer. The propagated plasmids were purified using Qiagen midiprep (Qiagen) and then applied on agarose gel in order to estimate the purity and concentration of plasmid. The concentrations were then finally determined by OD-spectrometry for each plasmid.

Transfection

Transfection of COS-7 and N2A cells and murine fibroblasts C3H 10T½. PEI solution was dialysed by dialysis membrane (MW-cutoff 15 kDa) in order to remove smaller PEI fragments that can be toxic to the cells. Thereafter TP10 was crosslinked to PEI as

described above. The PEI and/or TP10-PEI stock solutions (1 mg/ml) were diluted so that the same volume of solution could be taken for each N/P ratio experiment. 10µl of plasmid (0.05 µg/µl) and 10 µl of transfection reagent were mixed in a 96 well plate, each experimental point separately. The mixture was incubated 15 min at room temperatures. Meanwhiles media of 24 hours prior seeded cells was changed to fresh (250 µl/well in 24 well plate). After incubation of plasmid and PEI was finished, 20 µl of the conjugate was added to cells. Transfection was carried out for 3 h at 37 °C, followed by media change. Efficiency of transfection was measured 24 or 72 h after transfection.

Fluorescense microscopy

The level of GFP expression was investigated by an inverted fluorescence microscope (Zeiss Axiovert 200 equipped for fluorescence microscopy, or Olympus IMT2 inverted microscope with RFL-1 fluorescence device), 2-3 days after transfection.

GFP quantification

For GFP quantification in murine fibroblasts C3H 10T1/2 cells were lysed and the lysate was exitated at 485 nm, and UV emission was measured at 527 nm using Labsystems. Fluoroscan Ascent CF instrument (Labsystems, FI).

Luciferase assay

Cells transfected by renilla or firefly luciferase gene were lysed 36 hours after transfection using passive lysis buffer (Promega). Samples were freeze-thawed once and luciferase activity was measured on the same or next day. Protocol of dual luciferase kit, recommended by manufacturer (Promega) was used on Victor (Wallac, Finland) luminometer. Obtained luciferase activities were transformed into activity per mg of total protein concentration. Determination of total protein concentration was performed by Bradford method.

In vivo transfection of chicken embryo

Eggs with developing chicken embryos (3 days) were opened and SMV-lacZ reporter genes were injected on embryo near neural tube. After 48 h embryos were removed and fixed for 1-2 hours in 2% paraformaldehyde, 0.25% glutaraldehyde, washed with PBST and stained with staining solution (9 ml spermidine, 1 ml of 2% Xgal in DMF 0.5ml of 165 mg/ml K-ferricyanide, 0.45 ml 210 mg/mL K-ferrocyanide) until blue color developed (2h).

Results

TP10-PEI and GFP

TP10-PEI constructs mediated considerably higher levels of GFP transfection compared to unmodified PEI protocol at all tested concentrations. The most significant increase was achieved at concentrations below 1 µg TP10-PEI per well. At higher concentrations, the difference between modified and unmodified PEI was still significant, but not so drastic. Fig. 27 A, B and C. TP10 demonstrate the effect of unmodified PEI at concentrations of

0.25, 0.5 and 1.0 µg per well, respectively. D, E and F in the same figure correspond to TP10 modified PEI at same concentrations.
In a parallel study, murine fibroblasts C3H 10T1/2 were used instead of N2A cells. The GFP expression was determined up to 14 times higher (ratio of PEI-TP 0.4 µg vs.DNA 1.2µg) as with PEI alone under given experimental conditions. In fluorescent microscopy, the number of eGFP expressing cells was significantly higher in the samples transfected with PEI-TP.

TP10-PEI and luciferase
Under optimal conditions, in full growth media, TP10 modified PEI (TP10/PEI molar ratio 5) mediated about an 100% increase in luciferase transfection efficiancy (se Fig. 27) Under nonoptimal conditions no significant positive effect was observed.

TP10/PEI molar ratio
Four different TP10/PEI ratios in the range of 1 to 100 molecules of TP10 per 1 polycationic molecule were tested. Results showed that the delivery yield is depending on the ratio. 5 TP10 per one PEI was found to be the best ratio, followed by 20. Still the optimal ratio is probably not exactly 5, but somewhere between 5 and 20. Ratios 1 and 100 had lowest effect.

In vivo transfection of chicken embryo
When SMV-lacZ alone or in complex with PEI was applied to the embryo, no expression of the reporter gene was observed. If SMV-lacZ was complexed with PEI-TP10 the reporter gene was expressed in a neural tube region as detected by specific staining. The B-galactosidase staining was strong and was distributed with equal intensity all over the neural tube region.

Example 10

Positional scanning of CPP within human 7TM receptors.
Human 7TM receptor sequences were downloaded from the swissprot/trmbl databases. The sequences were searched for CPPs of the indicated length (8,12 and 17 aa long). The possition of the start of the CPP in the protein was divided by the total length of the protein, plotted against the frequencey of occurense (fraction of CPPs). Here, a search window in the size of 17 aa produced the most hits. The four peaks in evidence correspond to the four intracellular parts of the 7TM receptors, with the largest peak corresponding to the third internal loop (IC3). It can be noted that the CPP functionality seem to correlate well, both with the topology of the 7TM receptorss, as well as with the proposed G protein activation sites.
See Fig.28.

LIST OF REFERENCES

1. Lindgren, M., Hällbrink, M., Prochiantz, A. & Langel, Ü. Cell penetrating peptides. *Trends Pharmacol. Sci.* **21**, 99-103 (2000).
2. Derossi, D., Chassaing, G. & Prochiantz, A. Trojan peptides: the penetratin system for intracellular delivery. *Trends Cell. Biol.* **8**, 84-87 (1998).
3. Derossi, D., Joliot, A.H., Chassaing, G. & Prochiantz, A. The third helix of the Antennapedia homeodomain translocates through biological membranes. *J. Biol. Chem.* **269**, 10444-10450 (1994).
4. Prochiantz, A. Homeodomain-derived peptides. In and out of the cells. *Ann N Y Acad Sci* **886**, 172-9 (1999).
5. Sandberg, M., Eriksson, L., Jonsson, J., Sjöström, M. & Wold, S. New chemical descriptors relevant for the design of biologically active peptides. A multivariate characterization of 87 amino acids. *J. Med. Chem.* **41**, 2481-2491 (1998).
6. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. Basic local alignment search tool. *J Mol Biol* **215**, 403-10. (1990).
7. Ma, H. & Diamond, S.L. Nonviral gene therapy and its delivery systems. *Curr Pharm Biotechnol* **2**, 1-17. (2001).
8. Garnett, M.C. Gene-delivery systems using cationic polymers. *Crit Rev Ther Drug Carrier Syst* **16**, 147-207 (1999).
9. Somiari, S. *et al.* Theory and in vivo application of electroporative gene delivery. *Mol Ther* **2**, 178-87. (2000).
10. Lee, R.J. & Huang, L. Lipidic vector systems for gene transfer. *Crit. Rev. Therap. Drug Carrier Syst.* **14**, 173-206 (1997).
11. Ropert, C. Liposomes as a gene delivery system. *Braz J Med Biol Res* **32**, 163-9. (1999).
12. Luo, D. & Saltzman, W.M. Enhancement of transfection by physical concentration of DNA at the cell surface. *Nat Biotechnol* **18**, 893-5. taf/DynaPage.taf?file=/nbt/journal/v18/n8/full/nbt0800_893.html taf/DynaPage.taf?file=/nbt/journal/v18/n8/abs/nbt0800_893.html (2000).
13. Felgner, P.L. *et al.* Nomenclature for synthetic gene delivery systems. *Hum Gene Ther* **8**, 511-2. (1997).
14. Gebhart, C.L. & Kabanov, A.V. Evaluation of polyplexes as gene transfer agents. *J Control Release* **73**, 401-16. (2001).
15. Boussif, O. *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* **92**, 7297-301. (1995).
16. Abdallah, B. *et al.* A powerful nonviral vector for in vivo gene transfer into the adult mammalian brain: polyethylenimine. *Hum Gene Ther* **7**, 1947-54. (1996).
17. Schatzlein, A.G. Non-viral vectors in cancer gene therapy: principles and progress. *Anticancer Drugs* **12**, 275-304. (2001).
18. Remy-Kristensen, A., Clamme, J.P., Vuilleumier, C., Kuhry, J.G. & Mely, Y. Role of endocytosis in the transfection of L929 fibroblasts by polyethylenimine/DNA complexes. *Biochim Biophys Acta* **1514**, 21-32. (2001).
19. Rozanov, D.V. *et al.* Mutation analysis of membrane type-1 matrix metalloproteinase (MT1- MMP). The role of the cytoplasmic tail Cys(574), the active site Glu(240), and

furin cleavage motifs in oligomerization, processing, and self-proteolysis of MT1-MMP expressed in breast carcinoma cells. *J Biol Chem* **276**, 25705-14. (2001).

20. Smith, L.E., Parks, K.K., Hasegawa, L.S., Eastmond, D.A. & Grosovsky, A.J. Targeted breakage of paracentromeric heterochromatin induces chromosomal instability. *Mutagenesis* **13**, 435-43. (1998).

21. Tang, W. *et al.* Development and evaluation of high throughput functional assay methods for HERG potassium channel. *J Biomol Screen* **6**, 325-31. (2001).

22. Hallbrink, M. *et al.* Cargo delivery kinetics of cell-penetrating peptides. *Biochim Biophys Acta* **1515**, 101-9. (2001).

23. Vidal, P. *et al.* Interactions of primary amphipathic vector peptides with membranes. Conformational consequences and influence on cellular localization. *J Membr Biol* **162**, 259-64. (1998).

24. Kilk, K. *et al.* Cellular internalization of a cargo complex with a novel peptide derived from the third helix of the islet-1 homeodomain. Comparison with the penetratin peptide. *Bioconjug Chem* **12**, 911-6. (2001).

25. Magzoub, M., Kilk, K., Eriksson, L.E., Langel, U. & Graslund, A. Interaction and structure induction of cell-penetrating peptides in the presence of phospholipid vesicles. *Biochim Biophys Acta* **1512**, 77-89. (2001).

26. Inoue, A., Takahashi, M., Hatta, K., Hotta, Y. & Okamoto, H. Developmental regulation of islet-1 mRNA expression during neuronal differentiation in embryonic zebrafish. *Dev Dyn* **199**, 1-11. (1994).

27. Langel, U., Land, T. & Bartfai, T. Design of chimeric peptide ligands to galanin receptors and substance P receptors. *Int J Pept Protein Res* **39**, 516-22. (1992).

28. McKenzie, F.R. *Signal Transduction* (Milligan, E., Ed.), Oxford University Press, Oxford, NY, Tokyo. (1992).

29. Lowry, O.H., Rosenbrough, n.Y., Farr, A.L. & Randall, R.J. *J. Biol. Chem.* **193**, 265-275 (1951).

30. Cassel, D. & Selinger, Z. (1976) **452**(2), 538-51. **452**, 538-551 (1976).

CLAIMS

1. Method for identifying a cell-penetrating peptide or protein and/or a cell penetrating fragment of a peptide or protein, the method comprising the steps of

a) obtaining the amino acid sequence of said protein or peptide,
b) selecting the amino acid sequence of at least one candidate fragment,
c) assessing the bulk property value Z_Σ of said sequence, Z_Σ comprising at least 5 individual average interval values $Z_{\Sigma 1}$; $Z_{\Sigma 2}$; $Z_{\Sigma 3}$; $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$,

wherein $Z_{\Sigma 1}$, $Z_{\Sigma 2}$, $Z_{\Sigma 3}$, $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$ are average values of the respective descriptor values for the residues in said amino acid sequence, calculated with the formula

$$Z_{\Sigma x} = (Z_{xres1} + Z_{xres2} \ldots + Z_{xresn})/n$$

Z_{xresy} being the respective descriptor value for amino acid residue y comprised in the selected candidate fragment, and wherein the descriptor value of each residue corresponds to a Z_1, Z_2, Z_3, Z_4, and Z_5 descriptor value in a descriptor value scale as listed in **table 1A**, and

d) identifying a cell penetrating fragment from said at least one candidate fragment(s) based on its Z_Σ bulk property value,

a cell penetrating fragment being characterised by having a Z_Σ bulk property value essentially consisting of individual average interval values, wherein $Z_{\Sigma 1}<0.2$; $Z_{\Sigma 2}<1.1$; $Z_{\Sigma 3}<-0.49$; $Z_{\Sigma 4}<0.33$; and $Z_{\Sigma 5}<0.95$ and $Z_{\Sigma 5}>0.12$,

e) optionally verifying the cell-penetrating capacity of said identified peptide or protein and/or said fragment by *in vitro* and/or *in vivo* methods.

2. Method for checking cellular penetration properties of a peptide, the method comprising the
steps of

a) obtaining the amino acid sequence of the peptide,
b) assessing the bulk property value Z_Σ of said sequence, Z_Σ comprising at least 5 individual average interval values $Z_{\Sigma 1}$; $Z_{\Sigma 2}$; $Z_{\Sigma 3}$; $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$,

wherein $Z_{\Sigma 1}$, $Z_{\Sigma 2}$, $Z_{\Sigma 3}$, $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$ are average values of the respective descriptor values for the residues in said amino acid sequence, calculated with the formula

$$Z_{\Sigma x} = (Z_{xres1} + Z_{xres2} \ldots + Z_{xresn})/n$$

Z_{xresy} being the respective descriptor value for amino acid residue y comprised in the selected candidate fragment, and wherein the descriptor value of each residue corresponds to a Z_1, Z_2, Z_3, Z_4, and Z_5 descriptor value in a descriptor value scale as listed in **table 1A**, and

c) checking the cell penetrating properties of said peptide based on its Z_Σ bulk property value,

a cell penetrating fragment being characterised by having a Z_Σ bulk property value essentially consisting of individual average interval values, wherein $Z_{\Sigma 1}<0.2$; $Z_{\Sigma 2}<1.1$; $Z_{\Sigma 3}<-0.49$; $Z_{\Sigma 4}<0.33$; and $Z_{\Sigma 5}<0.95$ and $Z_{\Sigma 5}>0.12$,

e) synthesizing or isolating a peptide comprising the amino acid sequence of said identified cell-penetrating peptide, and

g) optionally verifying the protein-mimicking functionality and/or the cell-penetrating capacity of the synthesized or isolated peptide by *in vitro* and/or *in vivo* methods.

3. Method for producing a cell penetrating and functional protein-mimicking peptide, the method comprising the steps of

a) selecting a functional protein of interest,

b) obtaining the amino acid sequence of said selected protein,

c) selecting the amino acid sequence of at least one candidate fragment corresponding to an intracellular part of said protein,

d) assessing the bulk property value Z_Σ of said sequence, Z_Σ comprisisng at least 5 individual average interval values $Z_{\Sigma 1}$; $Z_{\Sigma 2}$; $Z_{\Sigma 3}$; $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$,

wherein $Z_{\Sigma 1}$, $Z_{\Sigma 2}$, $Z_{\Sigma 3}$, $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$ are average values of the respective descriptor values for the residues in said amino acid sequence, calculated with the formula

$$Z_{\Sigma x} = (Z_{xres1} + Z_{xres2} \ldots + Z_{xresn})/n$$

Z_{xresy} being the respective descriptor value for amino acid residue y comprised in the selected candidate fragment, and wherein the descriptor value of each residue corresponds to a Z_1, Z_2, Z_3, Z_4, and Z_5 descriptor value in a descriptor value scale as listed in **table 1A**, and

e) identifying a cell penetrating fragment from said at least one candidate fragment(s) based on its Z_Σ bulk property value,

a cell penetrating fragment being characterised by having a Z_Σ bulk property value essentially consisting of individual average interval values, wherein $Z_{\Sigma 1}<0.2$; $Z_{\Sigma 2}<1.1$; $Z_{\Sigma 3}<-0.49$; $Z_{\Sigma 4}<0.33$; and $Z_{\Sigma 5}<0.95$ and $Z_{\Sigma 5}>0.12$,

f) synthesizing or isolating a peptide comprising the amino acid sequence of said identified cell-penetrating peptide, and

g) optionally verifying the protein-mimicking functionality and/or the cell-penetrating capacity of the synthesized or isolated peptide by *in vitro* and/or *in vivo* methods.

4. Method for producing an artificial cell penetrating and/or an artificial cell penetrating and functional protein-mimicking peptide, the method comprising the steps of

a) designing at least one artificial peptide and/or peptide fragment,

b) assessing the bulk property value Z_Σ of the amino acid sequence of said artificial peptide or peptide fragment, Z_Σ comprisisng at least 5 individual average interval values $Z_{\Sigma 1}$; $Z_{\Sigma 2}$; $Z_{\Sigma 3}$; $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$,

wherein $Z_{\Sigma 1}$, $Z_{\Sigma 2}$, $Z_{\Sigma 3}$, $Z_{\Sigma 4}$ and $Z_{\Sigma 5}$ are average values of the respective descriptor values for the residues in said amino acid sequence, calculated with the formula

$$Z_{\Sigma x}= (Z_{xres1}+Z_{xres2}...+Z_{xresn})/n$$

Z_{xresy} being the respective descriptor value for amino acid residue y comprised in the selected candidate fragment, and wherein the descriptor value of each residue corresponds to a Z_1, Z_2, Z_3, Z_4, and Z_5 descriptor value in a descriptor value scale as listed in **table 1A**, and

e) checking the cell penetrating properties of said artificial peptide and/or peptide fragment based on its Z_Σ bulk property value,

a cell penetrating fragment being characterised by having a Z_Σ bulk property value essentially consisting of individual average interval values, wherein $Z_{\Sigma 1}$<0.2; $Z_{\Sigma 2}$<1.1; $Z_{\Sigma 3}$<-0.49; $Z_{\Sigma 4}$<0.33; and $Z_{\Sigma 5}$<0.95 and $Z_{\Sigma 5}$>0.12,

f) synthesizing said peptide and/or peptide fragment comprising the amino acid sequence identified as cell penetrating, and

g) optionally verifying the protein-mimicking functionality and/or the cell-penetrating capacity of the synthesized peptide and/or peptide fragment by *in vitro* and/or *in vivo* methods.

5. A method according to any of claims 1 to 3, wherein said protein is a transmembranal protein.

6. A method according to claim 5, wherein said protein is a protein selected from the group consisting of human PrpC, bovine PrpC, amyloid precursor protein (APP) and presenilin-1 (PS-1).

7. A method according to claim 5, wherein said protein is a mammalian receptor, such as a receptor belonging to the superfamilly of tyrosine kinase receptors, a 7TM recptor and/or a G-protein coupled receptor.

8. A method according to claim 7, wherein said protein is a protein selected from the group consisting of the GLP-1 receptor, AT1A receptor, and Dopamine receptor.

9. A method according to any of the preceding claims, wherein the cell-penetrating capacity of said peptide and/or peptide fragment is verified by detecting a change in the membrane potential and/or membrane properties of a cell, monitored as the cellular uptake rate of a membrane potential sensitive dye into said cell after exposure to said peptide and/or peptide fragment.

10. A method according to claim 9, wherein said membrane potential sensitive dye is $DIBAC_4$.

11. A cell-penetrating peptide and/or a non-peptide analogue thereof obtained by a method according to any of the preceding claims.

12. A cell-penetrating peptide essentially consisting of a peptide obtained by a method according to any of the preceding claims.

13. A cell-penetrating peptide selected from a 12 to 50 amino acid residues long peptide or a fragment of a peptide corresponding to one of the amino acid sequences listed in SEQ ID NR. 1-150.

14. A cell-penetrating peptide according to claim 13, wherein the peptide is 14 to 30 amino acid residues long.

15. A cell-penetrating peptide according to claim 13, wherein the peptide is 16 to 20 amino acid residues long.

16. A cell-penetrating peptide selected from a 12 amino acid residues long peptide or a fragment of a peptide corresponding to one of the amino acid sequences listed in SEQ ID NR. 151-2684.

17. A cell-penetrating peptide selected from a 16 amino acid residues long peptide or a fragment of a peptide corresponding to one of the amino acid sequences listed in SEQ ID NR. 2685-6233.

18. A functional analogue of a cell-penetrating peptide according to any of claims 11 to 17.

19. A cell-penetrating peptide and/or a non-peptide analogue thereof being at least 75% identical to a cell-penetrating peptide and/or a non-peptide analogue thereof according to any of claims 11 to 17.

20. A cell-penetrating peptide and/or a non-peptide analogue thereof comprising a cell-penetrating peptide and/or a non-peptide analogue thereof according to any of claims 11 to 17.

21. A cell-penetrating peptide and/or a non-peptide analogue thereof according to any of claims 11-20, selected from the group consisting of peptides comprising the amino acid sequence IVIAKLKA and/or a cell membrane penetrating functional analogue thereof.

22. A cell-penetrating peptide and/or a non-peptide analogue thereof according to claim 21, comprising the amino acid sequence IVIAKLKANLMCKTCRLAK.

23. A cell-penetrating peptide and/or a non-peptide analogue thereof according to any of claims 11-22, wherein the peptide is coupled to a cargo.

24. A cell-penetrating peptide and/or a non-peptide analogue thereof according to claim 23, wherein the peptide is coupled to a cargo by a S-S bridge.

25. A cell-penetrating peptide and/or a non-peptide analogue thereof according to claim 23 or 24, wherein the cargo is a cellular effector.

26. A cell-penetrating peptide and/or a non-peptide analogue thereof according to any of claims 23 to 25, wherein the cargo is a pharmaceutically active component.

27. A cell-penetrating peptide and/or a non-peptide analogue thereof according to any of claims 23 to 26, wherein the cargo is selected from the group consisting of a small molecule, peptide, protein, saccharide, single and/or double stranded oligonucleotide, plasmid, antibiotic substance, cytotoxic and/or antiviral agent.

28. A cell-penetrating peptide and/or a non-peptide analogue thereof according to any of claims 23 to 27, wherein the cargo is a marker molecule.

29. A vector for transfecting a cell, the vector comprising
a) a nucleic acid component,
b) a polycation conjugate, and
c) a cell-penetrating peptide and/or a non-peptide analogue thereof,
wherein the average rate of transfection per cell at identical transfection conditions is enhanced by a factor of at least 2, compared to a vector comprising only components a) and b), or only components a) and c).

30. A vector according to claim 29, wherein said vector is used in a transient transfection and/or a stable transfection of a cell.

31. A vector according to claim 30, wherein said vector is used in an *in vivo* and/or in an *in vitro* transfection of a cell.

32. A vector according to claim 31, wherein said vector is used for a non-viral transfection of a cell.

33. A vector according to any of claims 29-32, wherein said polycation conjugate is polyethylene imine (PEI).

34. A vector according to any of claims 29-33, wherein said cell-penetrating peptide is a peptide or a peptide fragment according to any of claims 11-28.

35. A cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector according to any of claims 11-28 or 29-35, further characterised by being cell and/or cell-type and/or tissue specific.

36. A cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector according to claim 35, wherein said peptide and/or a non-peptide analogue thereof and/or vector selectively interacts with a cell surface protein, thus mediating the cell and/or cell-type and/or tissue specific cellular penetration.

37. A cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector according to claim 36, wherein said cell surface protein is over-expressed in said specific cell and/or cell-type and/or tissue.

38. A cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector according to claim 36 or 37, wherein said cell surface protein is selected from the group consisting of receptor tyrosine kinase type receptors, glycosphingolipids, CD44, erbB2, erbB3, and neuropeptide receptors.

39. A cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector according to claim 35, wherein said peptide and/or vector selectively interacts with an over-expressed cellular and/or extracellular protein, thus mediating the cell and/or cell-type and/or tissue specific cellular penetration.

40. A cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector according to claim 39, wherein said over-expressed protein is selected from the group consisting of agonists and antagonists to cell and/or cell-type and/or tissue specific recptors.

41. A cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector according to claim 39 or 40, wherein said over-expressed protein is selected from the group consisting of proteases, protease inhibitors and protease activators.

42. Use of a cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector according to any of claims 11-28 or 29-41 for the manufacture of a pharmaceutical composition.

43. A pharmaceutical composition manufactured according to claim 42.

44. Use of a cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector and/or a pharmaceutical composition according to any of claims 11-28, 29-41 or 43 for gene therapy.

45. Use of a cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector according to any of claims 11-28 or 29-41 for the manufacture of a pharmaceutical composition for gene therapy.

46. Use of a cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector according to any of claims 11-28 or 29-41 for the manufacture of a drug delivery system for transmembrane transport across an epithelial membrane, such as across the epithelium in the intestinal/buccal system, the mucosa in the mouth, lung, anus or nose, or the blood brain barrier of a mammal.

47. Use of a cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector, a pharmaceutical composition and/or a drug delivery system according to any of claims 11-28, 29-41, 43, or 46 for the manufacture of a pharmaceutical composition for treating and/or preventing a medical condition selected from the group consisting of

infectious diseases, diabetes type I, diabetes type II, Alzheimers Disease, Parkinssons Disease, cancer.

48. Method for treating a patient who suffers from a medical condition, the method comprising administering a pharmaceutical composition comprising a cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector, a pharmaceutical composition and/or a drug delivery system according to any of claims 11-28, 29-41, 43, or 46 to a patient in need thereof.

49. Method of treating a patient who suffers from a medical condition selected from the group consisting of diabetes type I and II, Alzheimers Disease, Parkinssons Disease, a prion disease, a cardiovascular disease, an infectious disease, disorders resulting from perturbed signal transduction, or cancer, the method comprising administering a pharmaceutical composition comprising a cell-penetrating peptide and/or a non-peptide analogue thereof and/or a vector , a pharmaceutical composition and/or a drug delivery system according to any of claims 11-28, 29-41, 43, or 46 is administered to a patient in need thereof.

ABSTRACT

The present invention relates to a method for predicting or designing, detecting, and/or verifying a novel cell-penetrating peptide (CPP) and to a method for using said new CPP and/or a novel usage of a known CPP for an improved cellular uptake of a cellular effector, coupled to said CPP. Furthermore, the present invention also relates to a method for predicting or designing, detecting and/or verifying a novel cell-penetrating peptide (CPP) that mimicks cellular effector activity and/or inhibits cellular effector activity. The present invention additionally relates to the use of said CPP for treating and/or preventing a medical condition and to the use of said CPP for the manufacture of a pharmaceutical composition for treating a medical condition.

Fig. 1

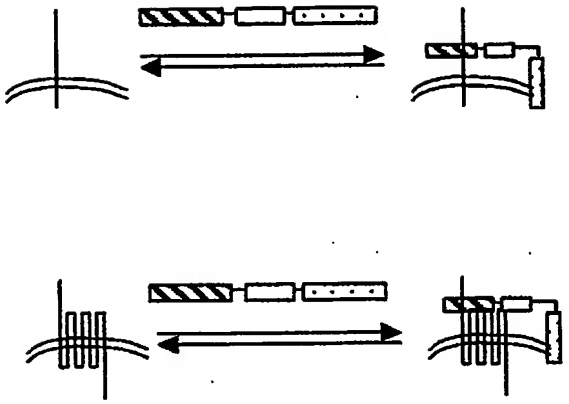


Fig. 2

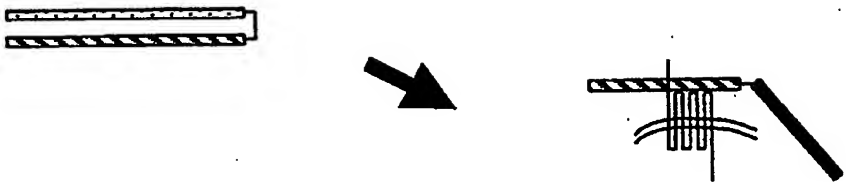


Fig. 3

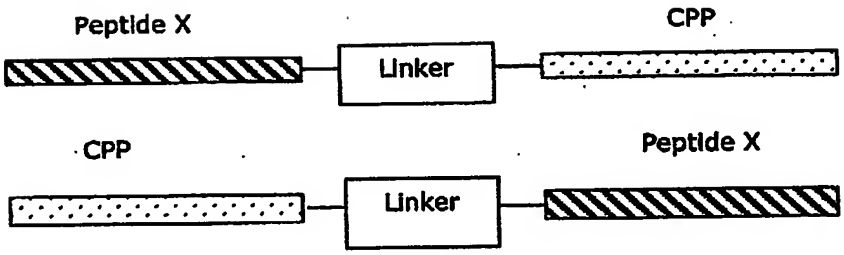


Fig. 4

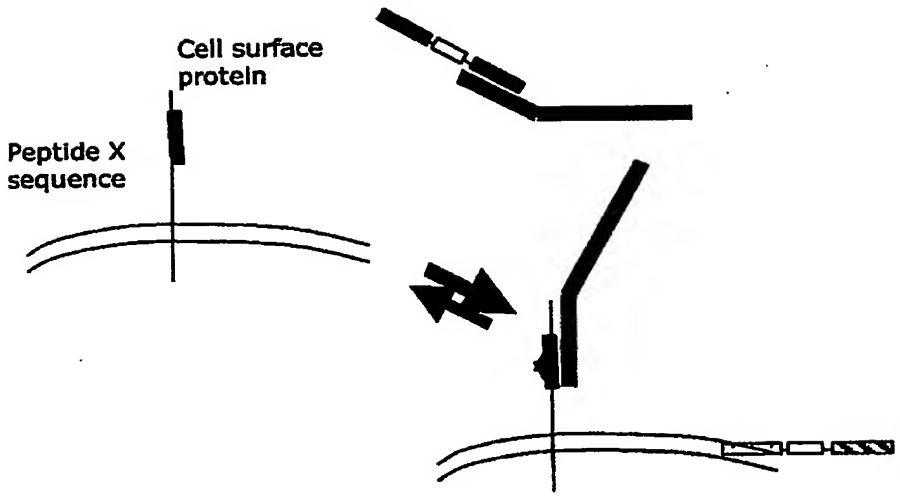


Fig. 5

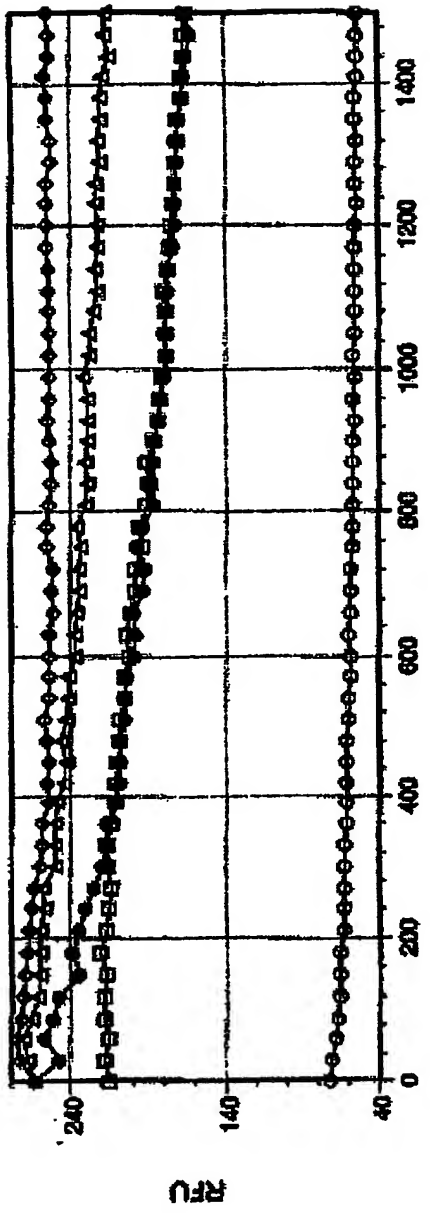


Fig. 3. Induction of DiBAC$_4$(3) cellular uptake by different penetratin derivatives

A2 Medium
B3 Cells with DiBAC$_4$(3)
D7 10 µM penetratin-aminobenzoic acid
B7 10 µM penetratin-biotin
F7 10 µM inactive penetratin

Fig. 6

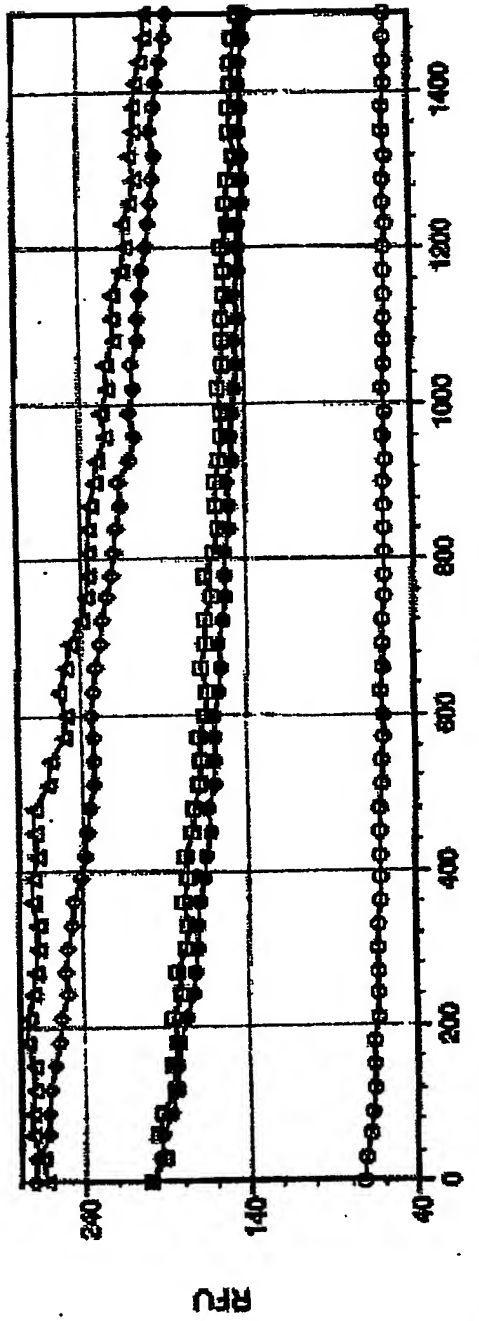


Fig. 1. Induction of DiBAC$_4$(3) cellular uptake by different transporter derivatives

A2 Medium
B1 Cells with DiBAC$_4$(3)
D2 10 µM transportan-aminobenzoic acid
F2 10 µM transportan-biotin
H1 10 µM inactive transportan

Fig. 7

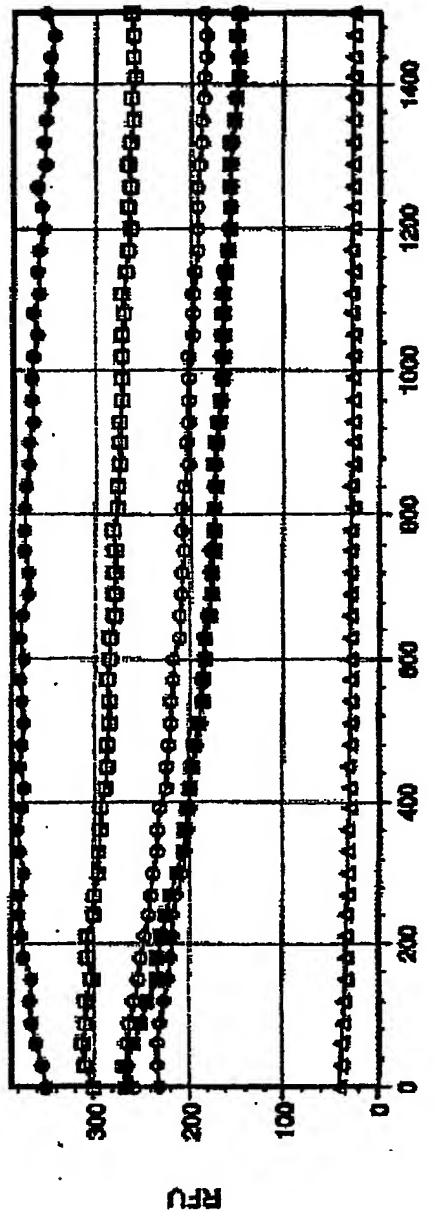


Fig. 2. Induction of DiBAC$_4$(3) cellular uptake is dependent on CPP concentration

H2 Medium
F2 Cells with DiBAC$_4$(3)
H2 1 μM transportan-biotin
B5 5 μM transportan-biotin
D5 10 μM transportan-biotin
F5 20 μM transportan-biotin

Fig. 8

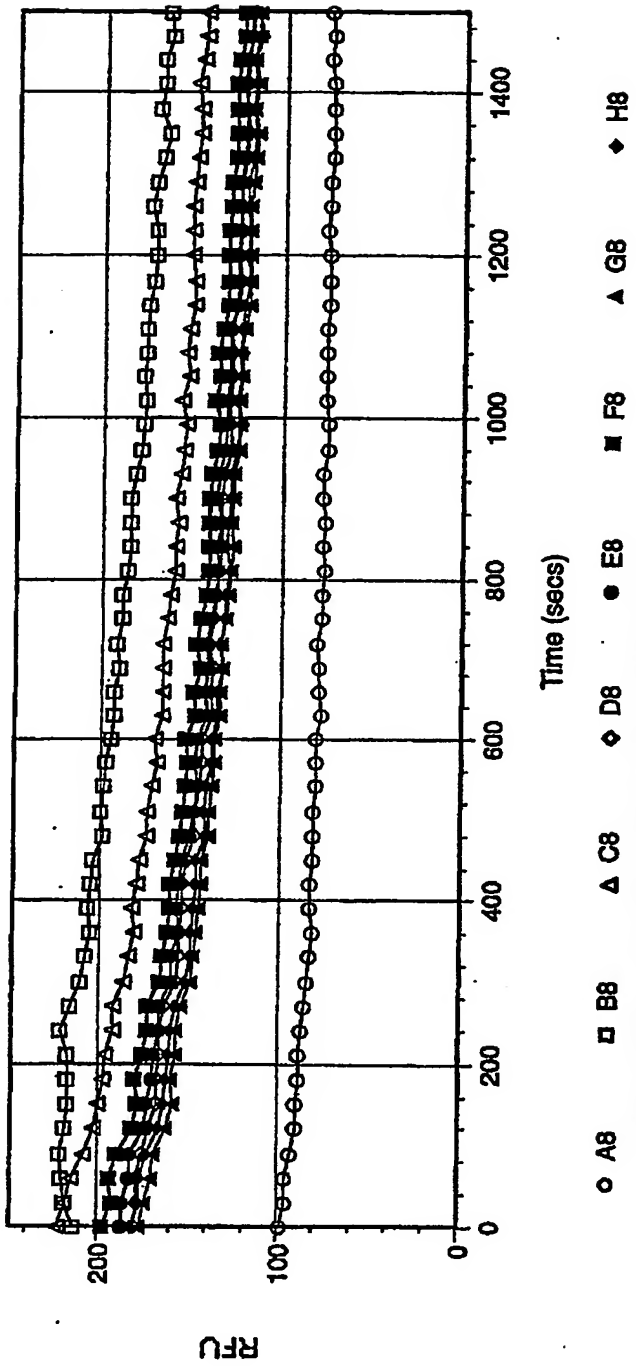


Fig. 4. Impact of pVEC on the uptake of $DiBAC_4(3)$

A8 Medium
B8 Cells with $DiBAC_4(3)$
C8 5 µM pVEC
D8 10 µM pVEC
E8 20 µM pVEC
F8 30 µM pVEC
G8 40 µM pVEC
H8 50 µM pVEC

Fig. 9

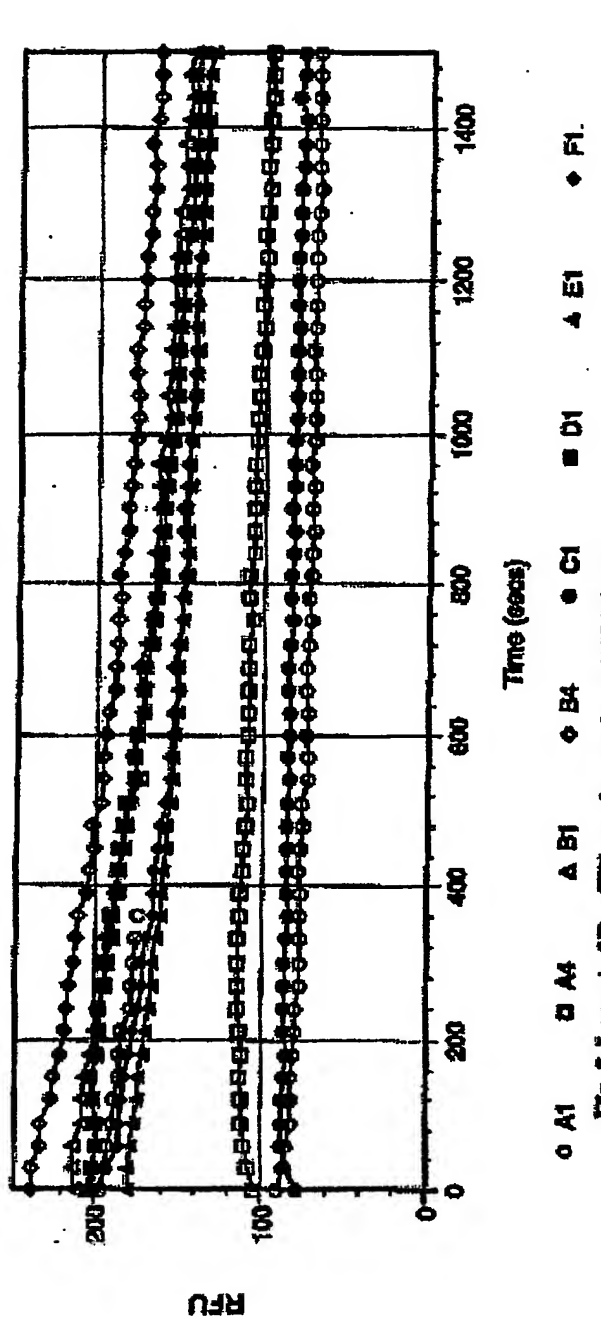


Fig. 5. Impact of Pro-TPb on the uptake of $DiBAC_4(3)$

A1 Medium
B1 Cells with $DiBAC_4(3)$
C1 5 µM Pro-TPb in medium
D1 5 µM Pro-TPb
E1 10 µM Pro-TPb
F1 20 µM Pro-TPb
G1 30 µM Pro-TPb
H1 40 µM Pro-TPb
A4 50 µM Pro-TPb in medium
B4 50 µM Pro-TPb

Fig. 10

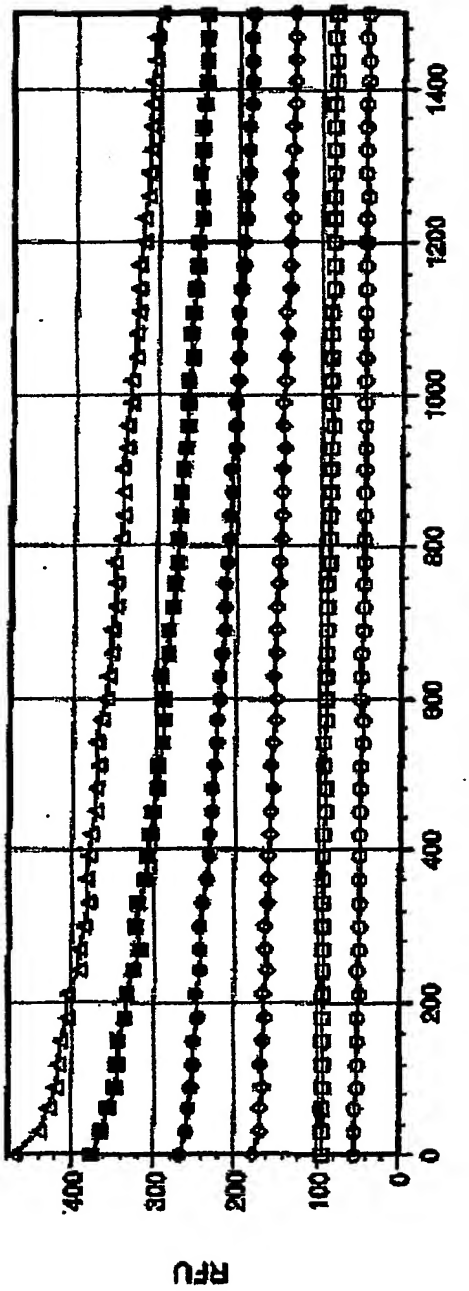


Fig. 6 Concentration-dependence of the fluorescence signal, $DiBAC_4(3)$

A2 0.2 µM $DiBAC_4(3)$ in medium
B2 0.2 µM $DiBAC_4(3)$
D2 0.4 µM $DiBAC_4(3)$
F2 0.6 µM $DiBAC_4(3)$
H2 0.8 µM $DiBAC_4(3)$
B5 1.0 µM $DiBAC_4(3)$

Fig. 11

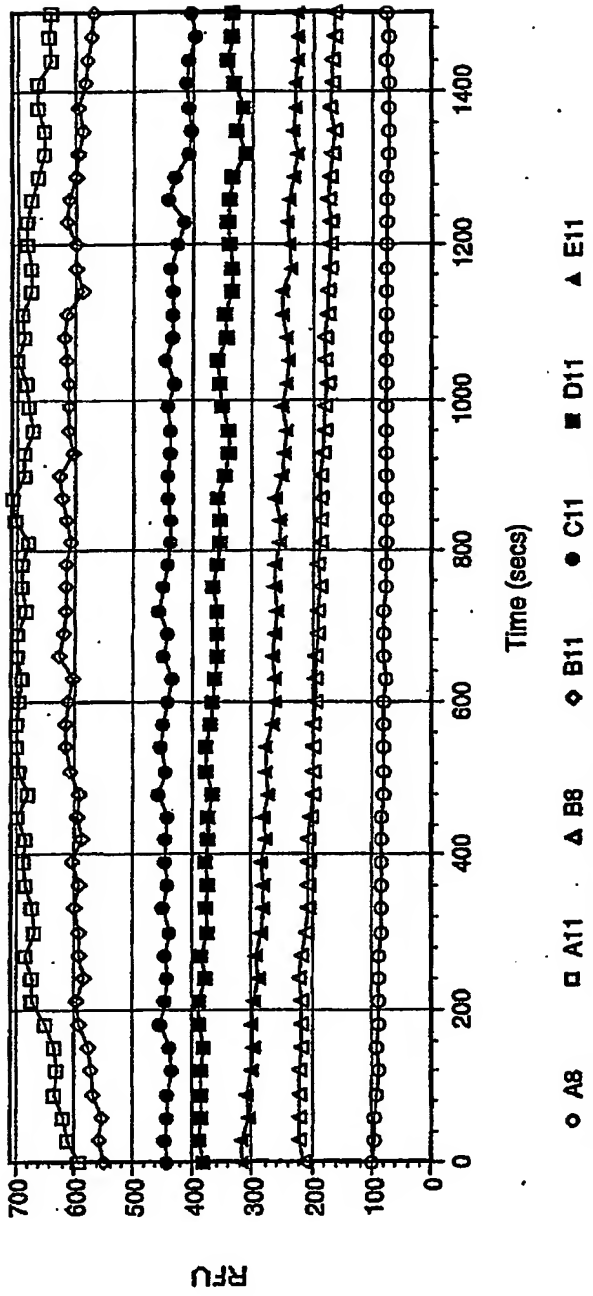


Fig. 7. Concentration-dependence of the fluorescence signal, peptide (TPb)

A8 Medium
C8 Cells with DiBAC$_4$(3)
B11 5 µM TPb
D11 10 µM TPb
C11 20 µM TPb
B11 30 µM TPb
A11 50 µM TPb

Fig. 12

Fig. 13

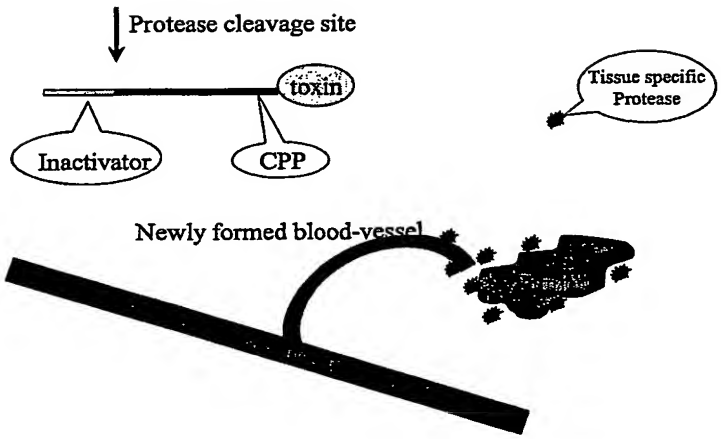

Protease cleavage site
toxin
Inactivator
CPP
Tissue specific Protease
Newly formed blood-vessel

Fig. 14

Fig. 15

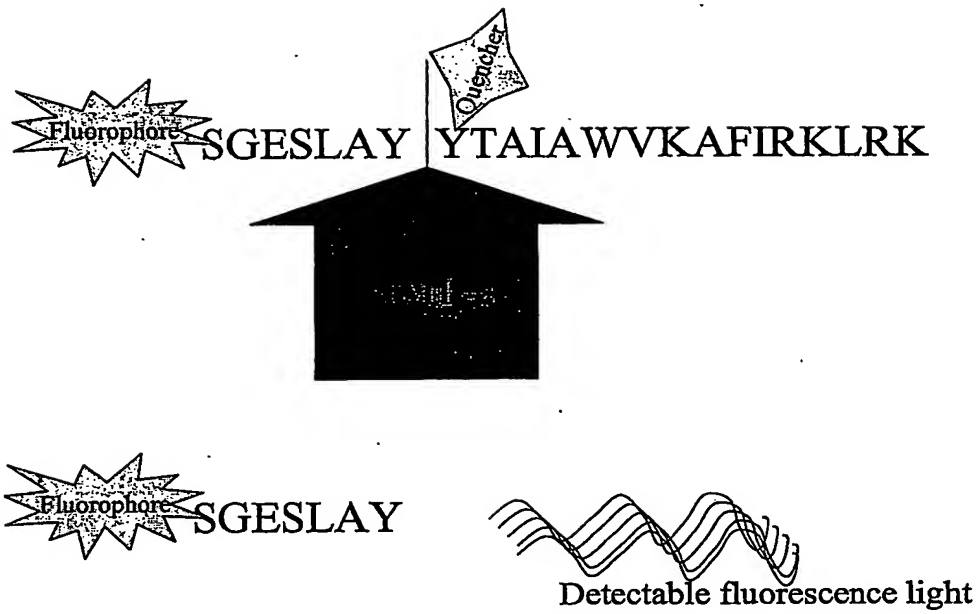

Fluorophore
SGESLAY
Quencher
YTAIAWVKAFIRKLRK
Fluorophore
SGESLAY
Detectable fluorescence light

Fig. 16

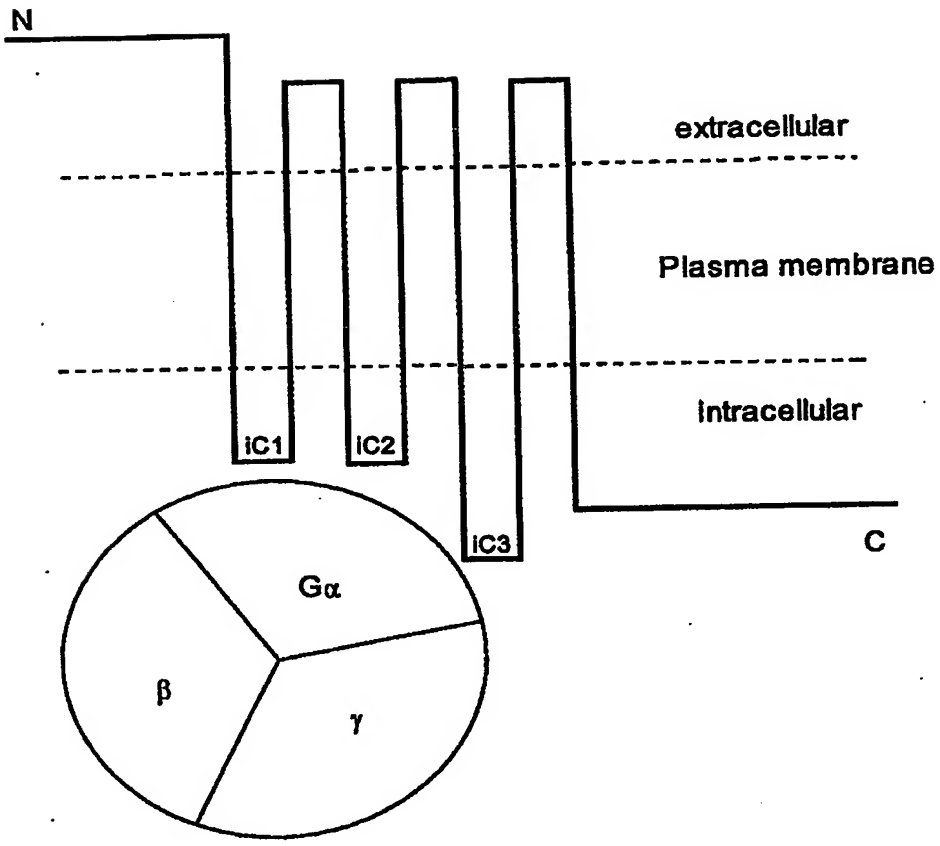

N
extracellular
Plasma membrane
intracellular
iC1
iC2
iC3
C
Gα
β
γ

Fig. 17

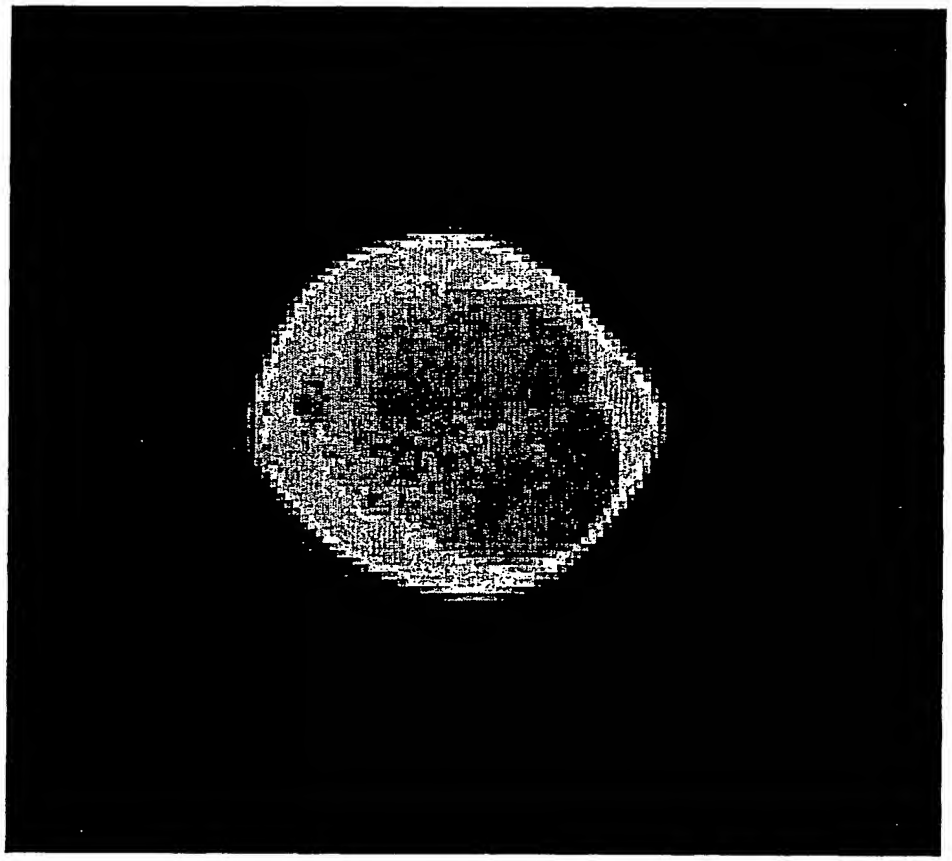


Fig 2. Cellular uptake of GOP (M569) in Bowes cells at 37oC

Fig. 18

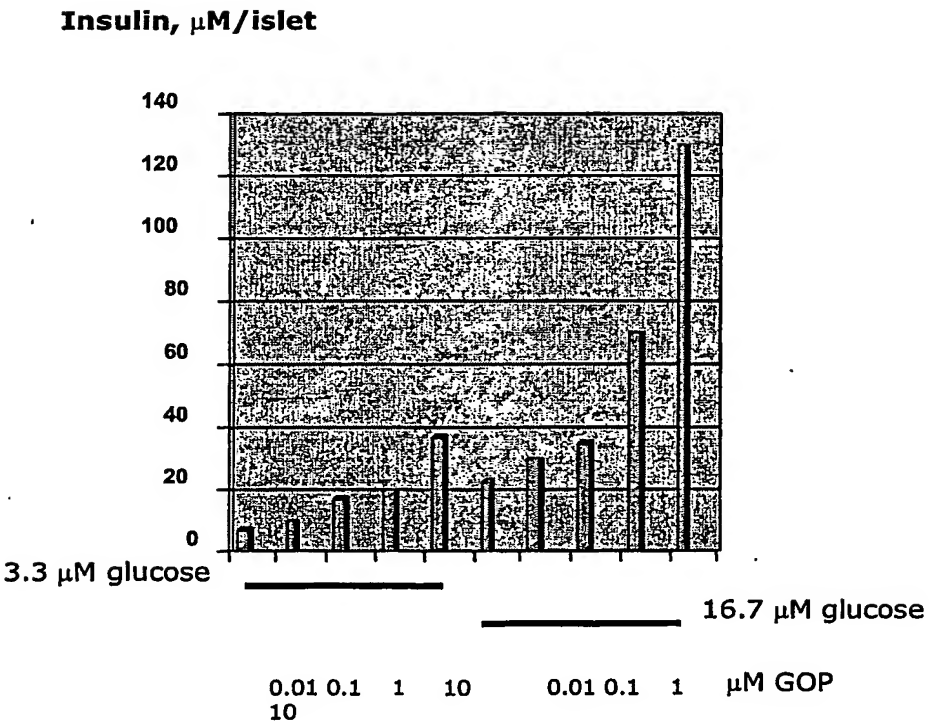

Insulin, µM/islet
140
120
100
80
60
40
20
0
3.3 µM glucose
16.7 µM glucose
0.01 0.1 1 10 0.01 0.1 1 10
µM GOP

Fig. 19 A

Blood glucose concentrations in healthy rats after i.p. injection of GOP (100 nmol/kg) and glucose (1 g/kg)

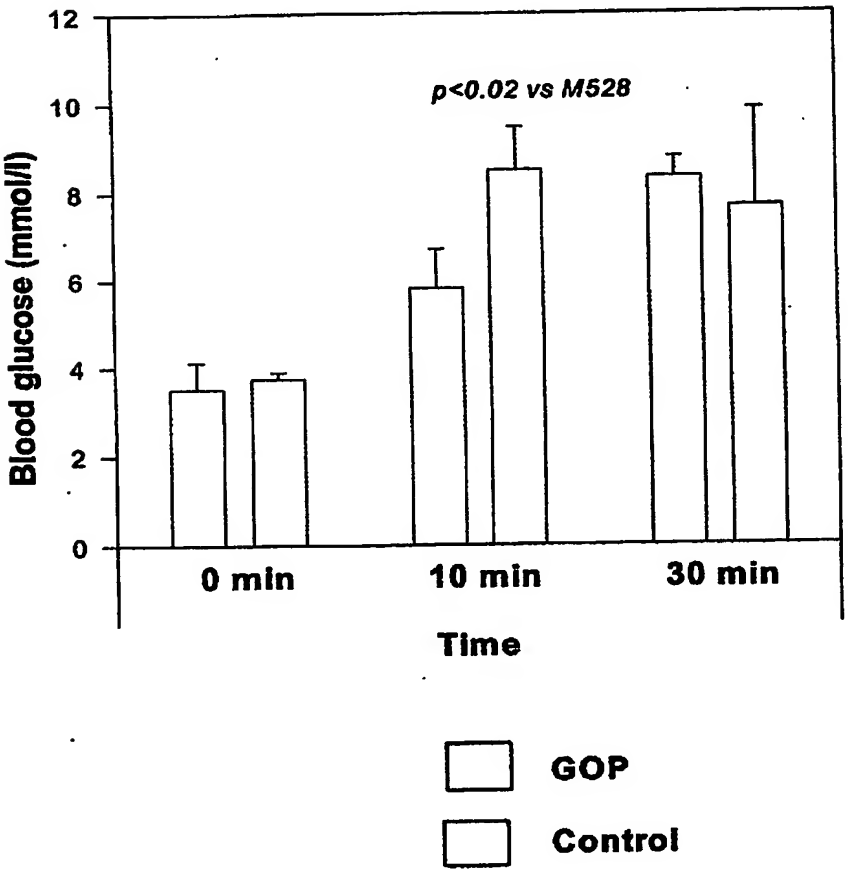


Fig. 19 B

Plasma insulin levels in healthy rats after i.p. injection of GOP (100 nmol/kg) and glucose (1 g/kg)

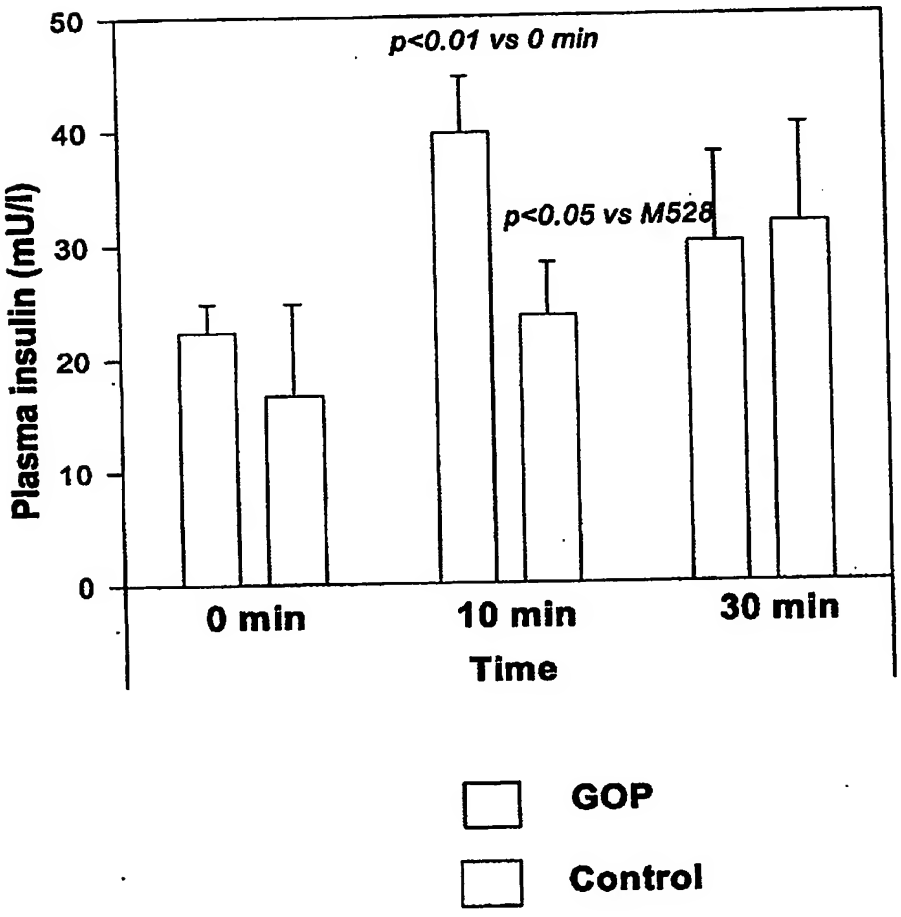


Fig. 20 A

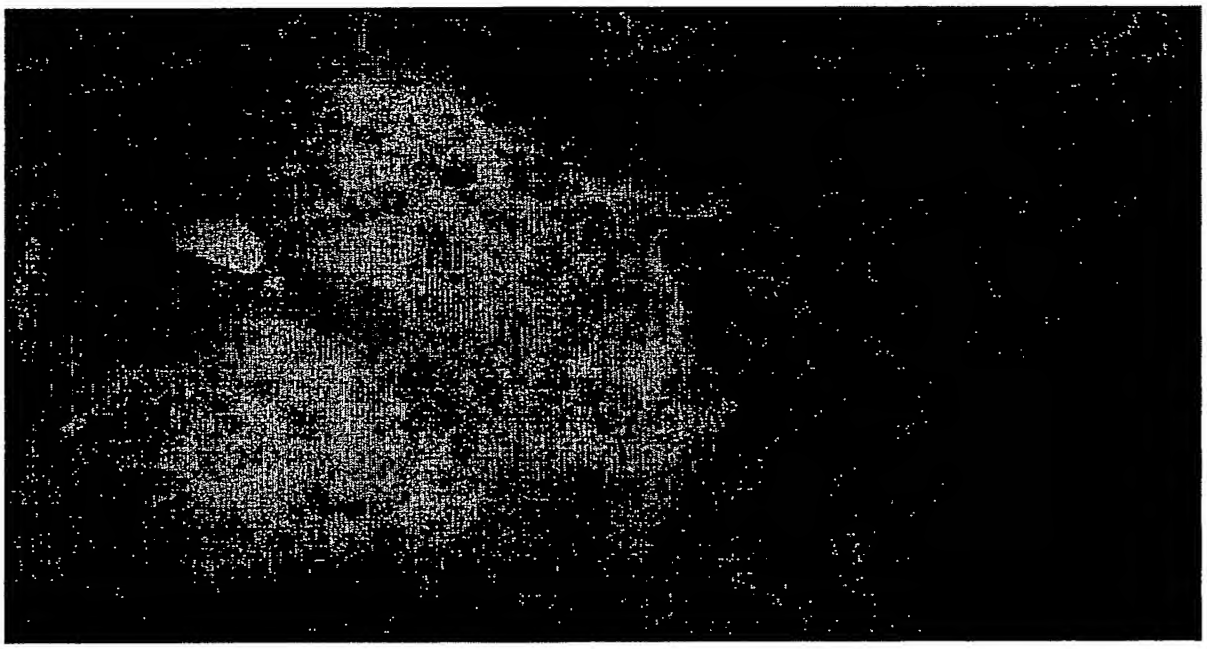


Fig. 20 B

YTA-2

Peptide:	Sequence:	Uptake
YTA-2	biotin-YTAIAWVKAFIRKLRK-amide	+++
YTA-2 ps	biotin-SGESLAYYTAIAWVKAFIRKLRK-amide	+

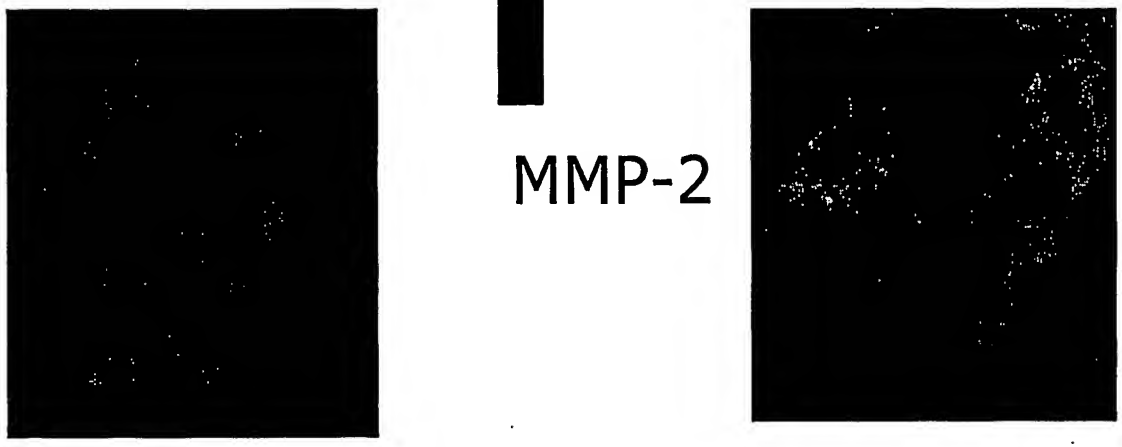
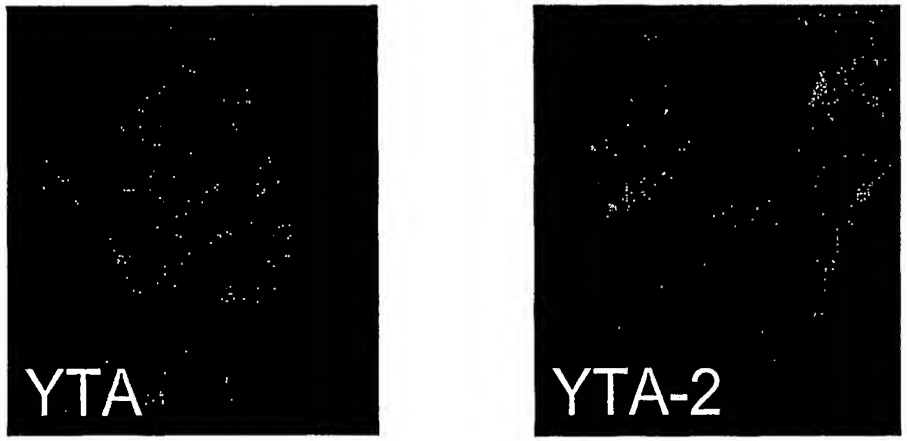


Fig. 20 C

YTA-2

Peptide	Sequenc	Translocatio efficienc
YTA2	bioti-YTAIAWVKAFIRK-amid	+++
YTA2 ps	bioti-SGESLAYYTAIAWVKAFIR-amid	+

inactiva



Translocation of 10µM peptide at 37 °C in LoVo cells

Fig. 21

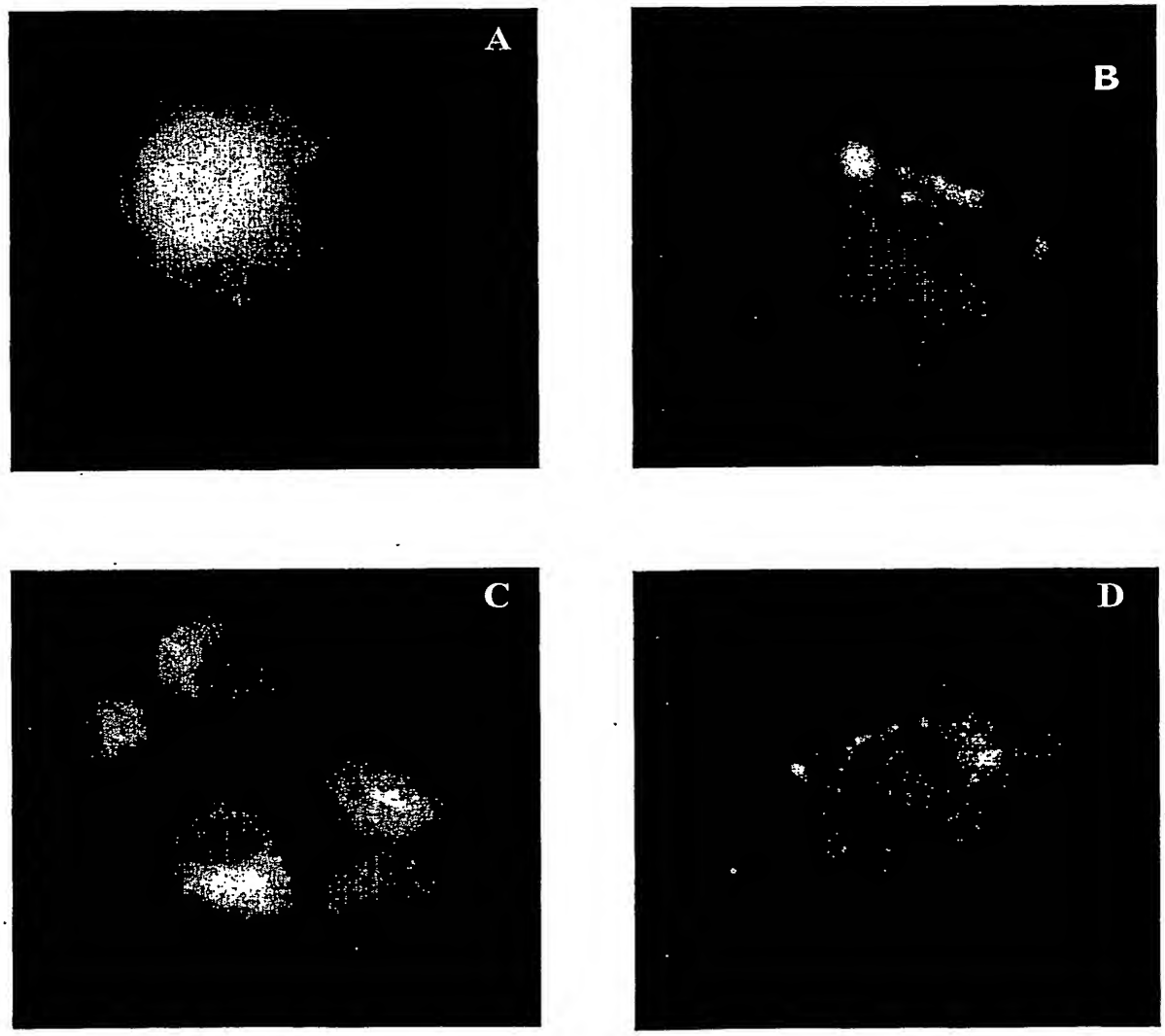


Fig. 22

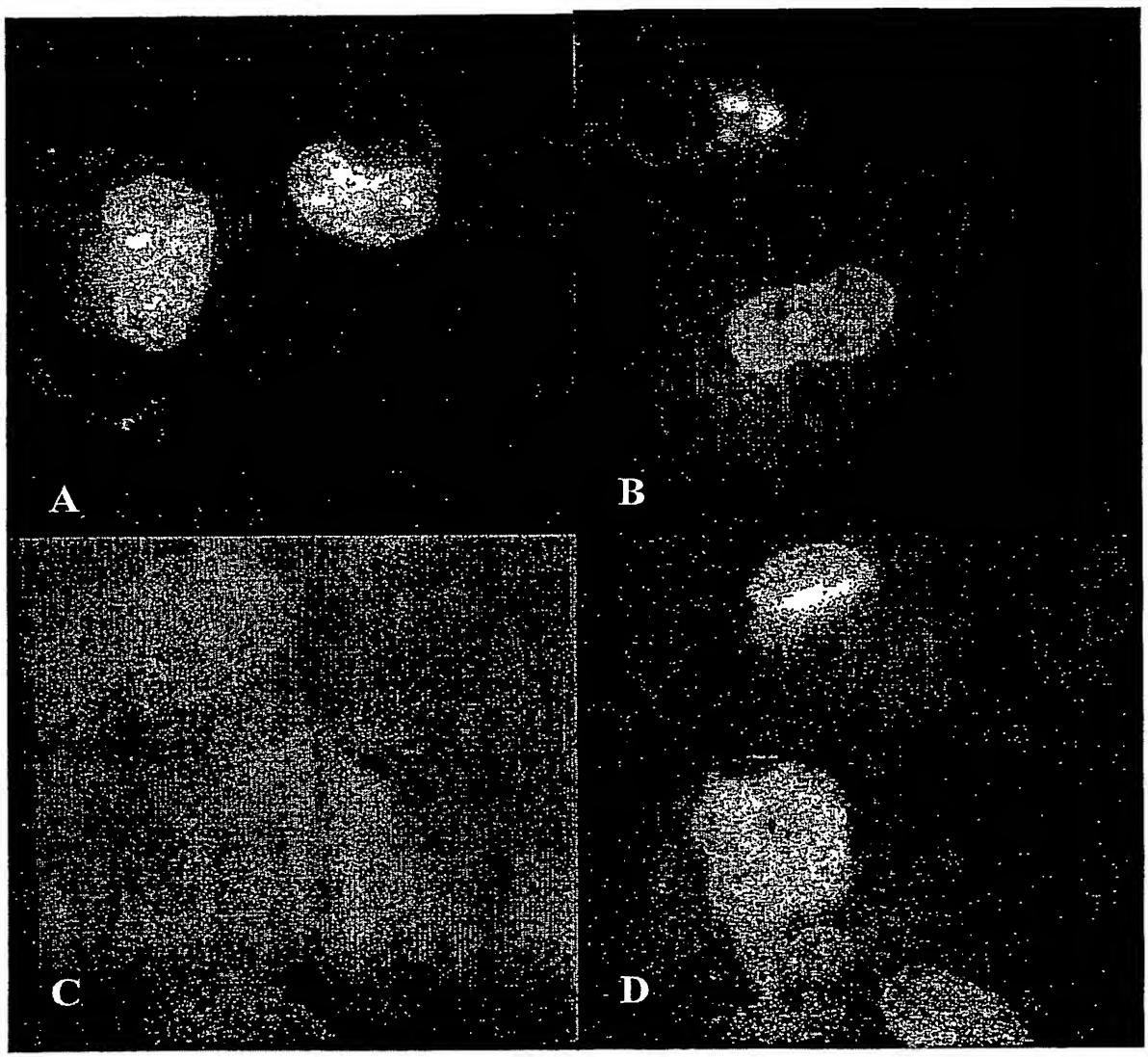


Fig. 23

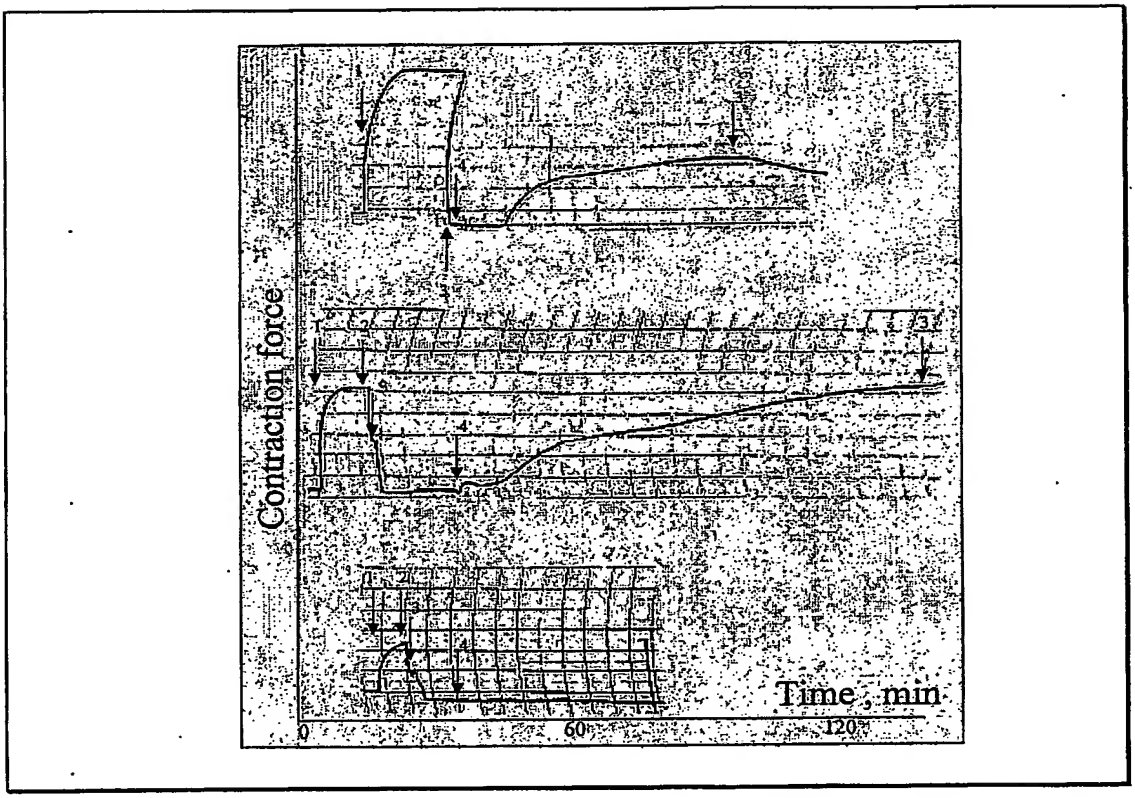


Fig. 24

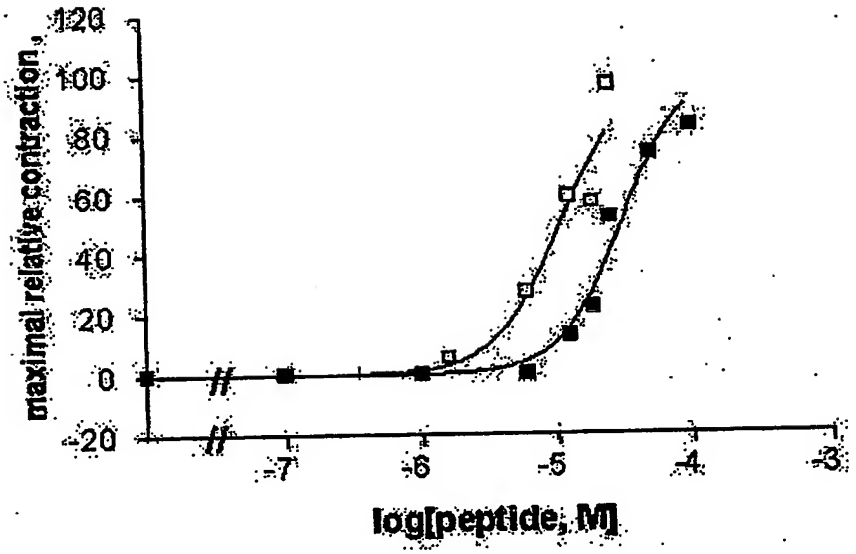

maximal relative contraction
120
100
80
60
40
20
0
-20
-7
-6
-5
-4
-3
log[peptide, M]

Fig. 25

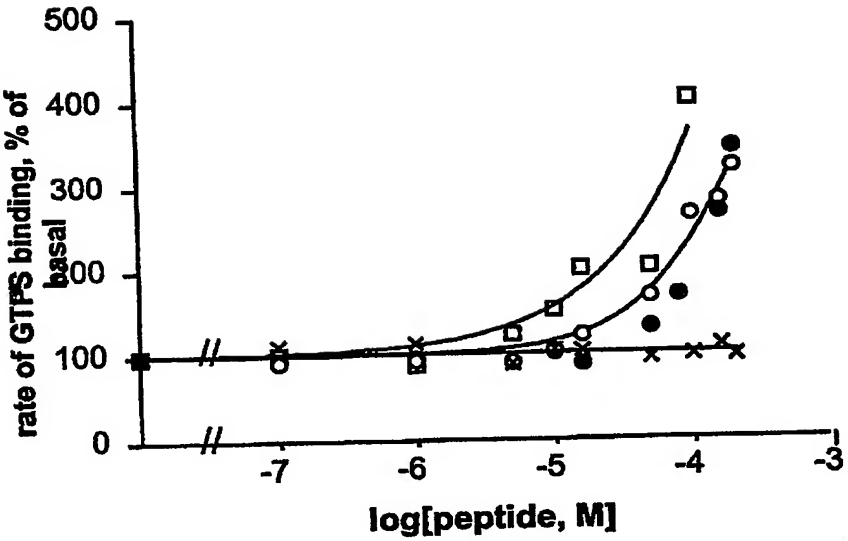

500
400
300
200
100
0
rate of GTPS binding, % of basal
-7
-6
-5
-4
-3
log[peptide, M]

Fig. 26

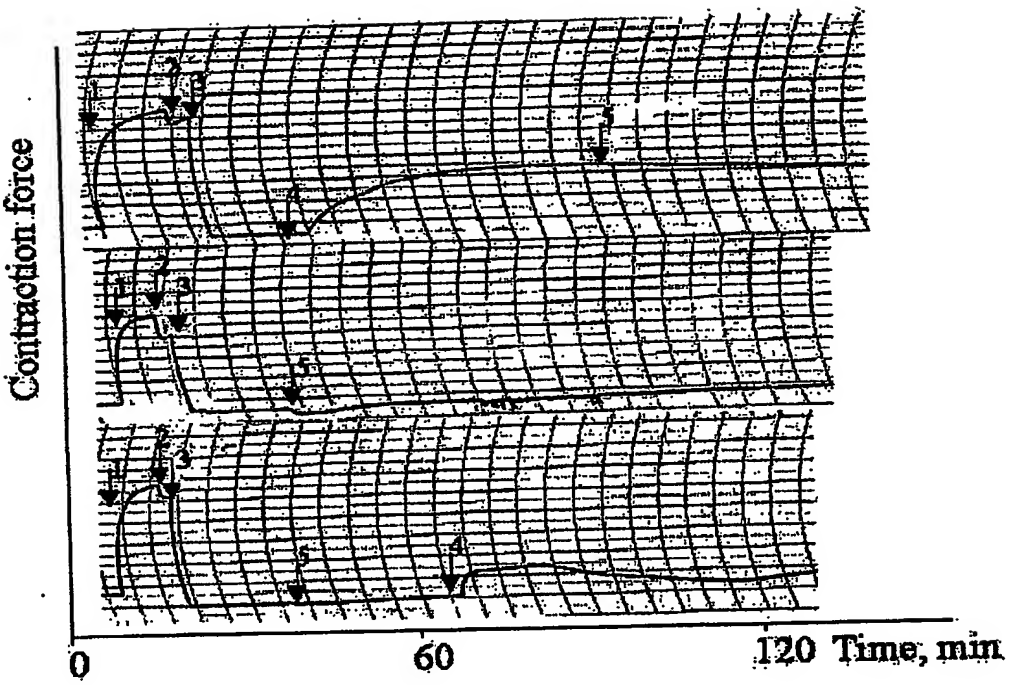


Fig. 27

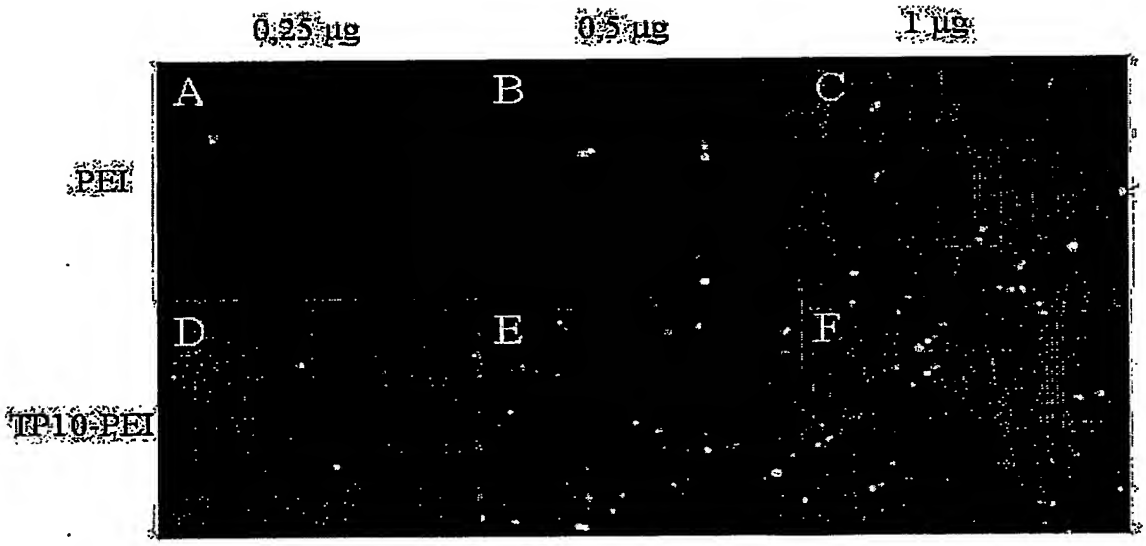


Fig. 27 B

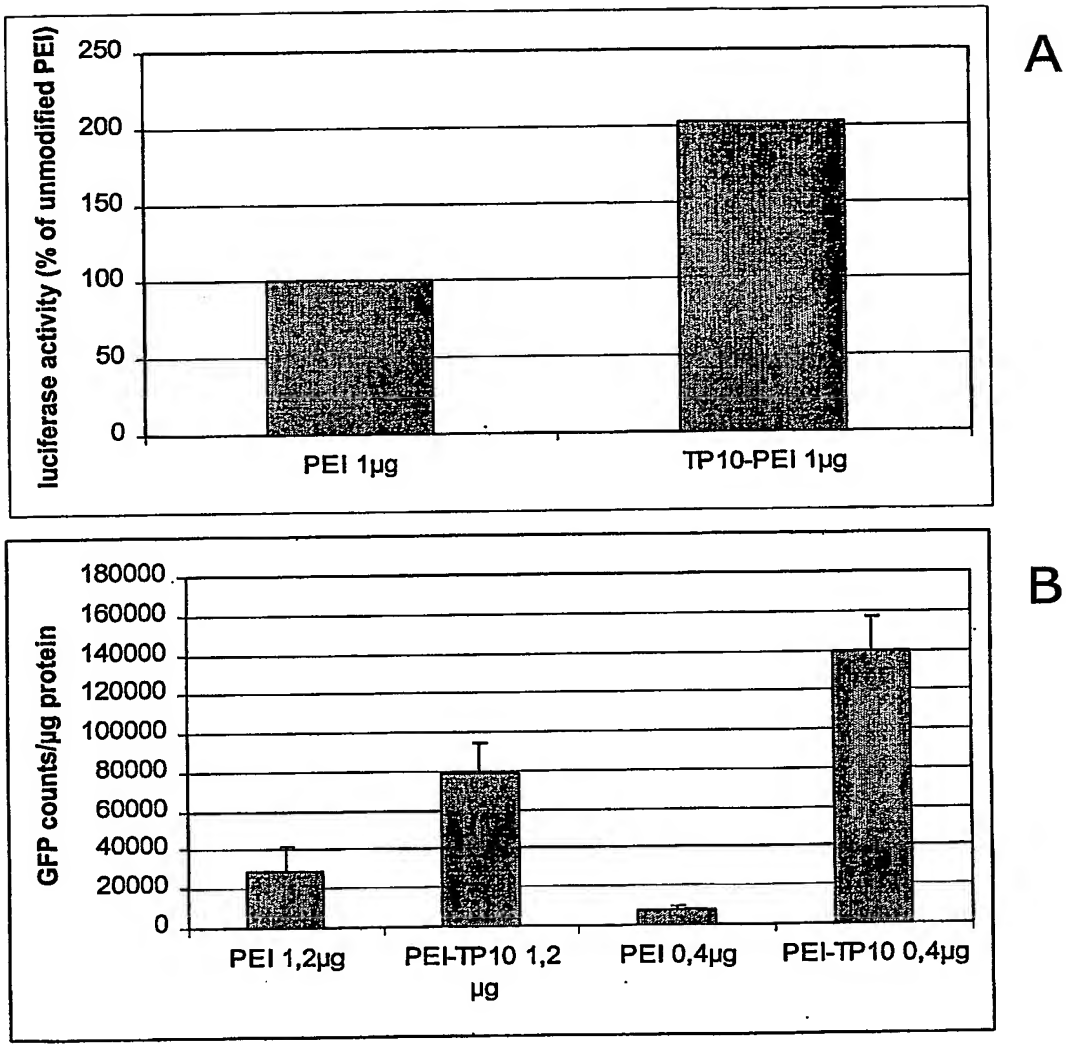
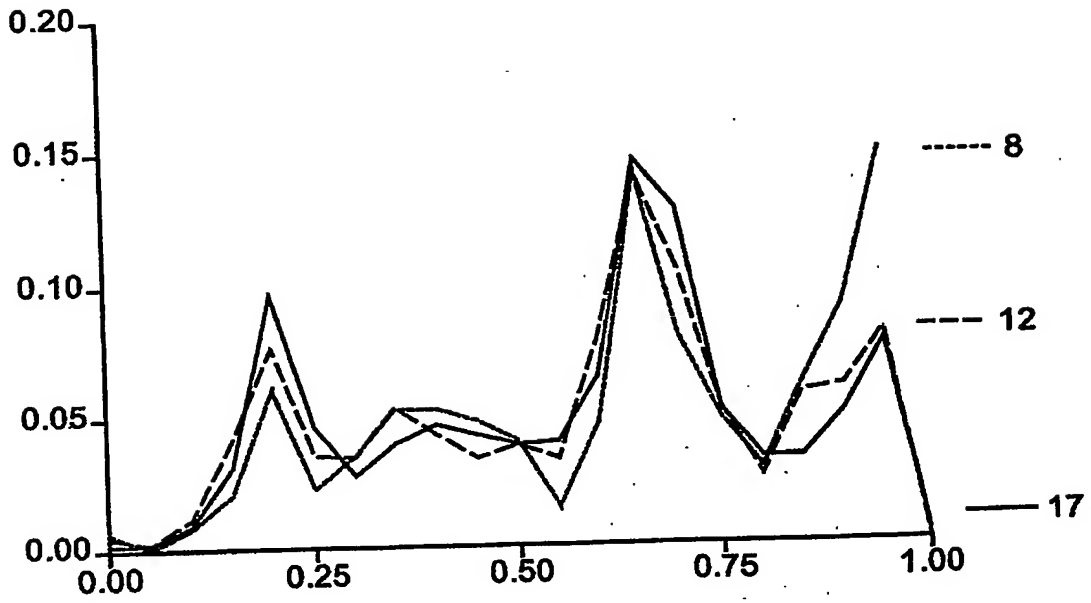

A
luciferase activity (% of unmodified PEI)
250
200
150
100
50
0
PEI 1µg
TP10-PEI 1µg
B
GFP counts/µg protein
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
PEI 1,2µg
PEI-TP10 1,2 µg
PEI 0,4µg
PEI-TP10 0,4µg

Fig. 28



BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☑ BLACK BORDERS
- ☑ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☑ FADED TEXT OR DRAWING
- ☑ BLURED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLORED OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☑ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REPERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning documents *will not* correct images problems checked, please do not report the problems to the IFW Image Problem Mailbox